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Unconventional mechanisms control cyclic respiratory gas release in flying *Drosophila*

Fritz-Olaf Lehmann* and Nicole Heymann

Department of Neurobiology, University of Ulm, Albert-Einstein-Allee 11, 89081 Ulm, Germany

*Author for correspondence (e-mail: fritz.lehmann@uni-ulm.de)

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Summary

The high power output of flight muscles places special demands on the respiratory gas exchange system in insects. In small insects, respiration relies on diffusion, and for elevated locomotor performance such as flight, instantaneous gas exchange rates typically co-vary with the animal's metabolic activity. By contrast, under certain conditions, instantaneous release rate of carbon dioxide from the fruit fly *Drosophila* flying in a virtual-reality flight arena may oscillate distinctly at low frequency (0.37±0.055 Hz), even though flight muscle mechanical power output requires constant metabolic activity. Cross-correlation analysis suggests that this uncoupling between respiratory and metabolic rate is not driven by conventional types of convective flow reinforcement such as abdominal pumping, but might result from two unusual mechanisms for tracheal breathing. Simplified analytical modeling of diffusive tracheal gas exchange suggests that cyclic release patterns in the insect occur as a consequence of the stochastically synchronized control of spiracle opening area by the four large thoracic spiracles. Alternatively, in-flight motion analysis of the abdomen and proboscis using infra-red video imaging suggests utilization of the proboscis extension reflex (PER) for tracheal convection. Although the respiratory benefit of synchronized spiracle opening activity in the fruit fly is unclear, proboscis-induced tracheal convection might potentially help to balance the local oxygen supply between different body compartments of the flying animal.

Key words: flight, respiration, discontinuous gas exchange cycle, spiracle modeling, insect, fruit fly, *Drosophila.*

Introduction

Over the past decades energy consumption and metabolic activity of animals have been vigorously investigated, especially with respect to circulation and respiration. In insects, tremendous progress has been made in identifying respiratory strategies and spiracle function in resting animals, whereas relatively few studies have addressed the mechanisms of respiratory gas exchange in running and flying insects (Bartholomew et al., 1985; Berrigan and Lighton, 1994; Harrison and Roberts, 2000; Herreid II and Full, 1984; Herreid II et al., 1981; Komai, 2001; Lehmann, 2001; Lighton, 1994, 1996; Miller, 1960; Wasserthal, 2001). During rest, many insects close the outer entrances of the tracheal system *via* spiracles and only exchange oxygen and carbon dioxide during brief periods of time. This behavior results in a discontinuous gas exchange cycle (DGC) that may limit both toxic oxygen levels in body tissues and respiratory water loss from insects living under xeric environmental conditions (Duncan and Byrne, 2002, 2005; Hadley, 1994; Hetz and Bradley, 2005; Lighton, 1994; Miller, 1981; Slama, 1994; Snyder et al., 1995). For example, respirometric recordings in the ant *Camponotus* have shown that water only leaves the tracheal system when the spiracles open for gas exchange (Lighton, 1992; Lighton and Garrigan, 1995). Similar results were obtained in resting fruit flies *Drosophila mimica* (Lehmann, 2000).

In most running insects, as energetic demands increase the DGC typically ceases, allowing respiratory gas exchange rates to increase likewise (Full and Tullis, 1990; Full et al., 1990; Jensen and Holm-Jensen, 1980; for reviews, see Lighton, 1994, 1996). In the desert ant *Pogonomyrmex rugosus*, Lighton and Feener (1989) reported a discontinuous breathing pattern while the unrestrained animal was walking voluntarily at constant speed within a respirometric chamber. A similar breathing pattern was found in blowflies *Protophormia terraenovae* walking back and forth in a running tube (Berrigan and Lighton, 1994). Although the first study was originally interpreted as a rare example in which the environment constrained breathing behavior to avoid excessive water loss, a recent study on walking energetics in ants suggests that DGC-like respiratory behavior in walking animals may also result from Doppler-effects occurring inside a running tube under flow-through respirometric conditions (Lipp et al., 2005).

In flying insects, flight-specific metabolic rate increases up to 15-fold over resting values and spiracles open in order to

allow gas exchange rates to increase (Casey, 1980, 1989; Casey and Ellington, 1989; Harrison and Roberts, 2000; Hedenström et al., 2001; Lehmann et al., 2000; Miller, 1960; Moffatt, 2001; Wasserthal, 2001; Weis-Fogh, 1972). In small animals such as the fruit fly *Drosophila*, the uptake of oxygen into and the release of carbon dioxide out of the tracheal system are thought to be a diffusion-based process, and thus the spiracle opening area matches the metabolic needs of the animal (Lehmann, 2001; Weis-Fogh, 1964). Applying the diffusive theory of respiration, adaptive spiracle control in *Drosophila* may potentially establish constant oxygen partial pressures near atmospheric partial pressure within the tracheae (Lehmann, 2001). Large insects additionally ventilate their tracheal air system to satisfy the increased oxygen needs of four distinct but interacting mechanisms: (i) contraction of the abdomen (abdominal pumping; for example, Harrison and Roberts, 2000; Komai, 2001; Miller, 1960); (ii) potentially, by muscle-induced deformations of large tracheae, as shown in insects breathing under X-ray in a synchrotron (Westneat et al., 2003); (iii) thoracic auto-ventilation resulting from the vibrations of the thorax during wing flapping (Miller, 1966); and (iv) directed Bernoulli suction-ventilation due to differences in static pressure distribution above two thoracic spiracles (Miller, 1966). In the hawkmoth *Manduca sexta*, for example, auto-ventilation produces pronounced pressure fluctuations in-phase with the 20 ms wing flapping cycle, causing inhalation during the downstroke and exhalation during the upstroke of respiratory gases on a stroke-by-stroke basis (Wasserthal, 2001).

In contrast to convective flow, diffusion-based respiratory gas exchange mechanisms for flight have in common that the instantaneously measured gas exchange rate is thought to reflect the animal's actual respiratory demands because there is no bulk flow of respiratory gases into and out of the tracheal system (Kestler, 1985). Experimentally, oxygen demands and the magnitude of CO_2 release rate from tethered *Drosophila* can be controlled by flying the animal in a respirometric chamber under visually controlled feed-back conditions (Dickinson and Lighton, 1995). In response to the vertical motion of a visual pattern displayed in a surrounding panorama, the animal modulates the muscle mechanical power output of its asynchronous flight muscles, and thus metabolic rate. When visual lift stimuli are absent, the rate with which the fly releases CO_2 changes only slightly because flight muscle mechanical power output, and thus metabolic rate, is not extensively modulated by the fly's nervous system.

In comparison to previous findings, we here report a novel type of gas release pattern in an insect by demonstrating that instantaneous CO_2 release rate of tethered flying *Drosophila* may periodically oscillate with large amplitudes, even though the metabolic rate of the animal remains approximately constant. In general, oscillatory gas release patterns in insects flying at constant metabolic rate may result from at least two distinct major mechanisms: ventilation and changes in the gas exchange area of the spiracles. While the first mechanism results from compression of air sacs and tracheae, the second one relies on spiracle control strategies. We thus evaluated the underlying physiological mechanisms of cyclic breathing behavior at an integrative level of investigation by combining (i) CO_2 release measurements of single fruit flies flying in a virtual-reality flight simulator with (ii) video-based in-flight tracking data of abdomen and proboscis movements, and (iii) employing an analytical model for tracheal gas diffusion through spiracles that allows simulations of tracheal CO_2 partial pressure changes and gas release rates at various metabolic rates.

Materials and methods

Respiratory measurements

The methods used in this study have been published elsewhere in greater detail (Lehmann, 2001; Lehmann and Dickinson, 1997) and we provide only a brief description here. Female *Drosophila melanogaster* Meigen (4–7 days old) from a laboratory colony were tethered and flown in a 15 ml flow-through respirometric chamber. Water vapor and CO_2 were removed from room air using a Drierite®/ascarite column, pulled at 1000 ml min^{-1} flow rate through the chamber using a mass-flow controller (El-Flow, Bronkhorst, Ak Ruurlo, Netherlands), and subsequently sampled in a gas analyzer (Licor-7000, Licor, Lincoln, USA). The internal filter frequency of the gas analyzer was set to 0.2 s. Data sampling frequency was 125 Hz and wash-out time constant τ of the respirometric chamber was approximately 910 ms (wash-out time = $ke^{-x/\tau}$). We estimated the time constant by inserting a small tube inside the respirometric chamber, through which we released small amounts of CO_2 similar to those released from a flying fly. Since the wash-out time constant depended exponentially on flow rate, a high flow rate yielded better temporal resolution but produced relatively low maximum gas concentrations, of approximately 1.0 p.p.m. CO_2. The signal-to-noise ratio (SNR) was approximately 18.6 dB. Flight muscle-specific mechanical power output was calculated from measurements of total flight force production, wing stroke amplitudes and stroke frequency according to energetic theory for flapping flight (Ellington, 1984; Lehmann and Dickinson, 1997). The respirometric chamber was surrounded by a computer-controlled cylindrical array of light-emitting diodes that allowed open- and closed-loop visual stimulation of the flying animal. Similar to previous procedures, we modulated the mechanical power output of the fly's flight muscle by oscillating a visual stripe grating in open-loop vertically around the fly, while the fly actively controlled the azimuth velocity of a single black stripe displayed in the arena (closed-loop conditions; Lehmann and Dickinson, 1997). All experiments were performed at approximately 28°C ambient temperature. For data analysis and modeling we employed self-written software routines in LabTalk (Origin 7.0, Microcal, Northampton, USA).

Infra-red video analysis

To evaluate abdominal pumping during flight of *Drosophila*, we developed an automatic tracking technique that allowed simultaneous recordings of abdominal length and width while the animal was flying inside the respirometric chamber. For this purpose, we marked the underside of the fly's abdomen with four small droplets of commercial yellow fluorescent dye and illuminated these markers using a UV-light emitting diode. While measuring wing kinematics and carbon dioxide release, we recorded in-flight movements of the abdomen using a conventional 50 Hz video camera (frame rate=20 ms). Subsequently, the positions of the four fluorescent markers were automatically tracked using a commercial video analysis program (MaxTraq, Innovision, Columbiaville, MI, USA). In-flight extensions of the fly's proboscis were derived by analyzing light intensity changes on the video images in a rectangular region (ROI) in front of the animal's head. To allow easy adjustments of ROI size and shape for each fly, we employed self-written software developed under Visual C++ and Matrox Imaging Library (Matrox, Quebeck, Canada).

Theoretical modeling of spiracle function

To assess the consequences of synchronized spiracle opening activity for cyclic gas release patterns, we modeled flight muscle-specific CO_2 release out of the tracheal system, assuming diffusive gas exchange between the muscle tissue, tracheal system and the ambient air (Kestler, 1985). Due to the large diffusive area of the four thoracic spiracles in *Drosophila* (9862 μm^2, ~95% of total spiracle area; Demerec, 1965), we excluded the 14 smaller abdominal spiracles from the theoretical modeling (Fig. 1A). We modeled the four spiracles as working units that independently control tracheal partial pressure of CO_2, $P_{T_{CO_2}}$, around a threshold value. In a diffusion-based system, $P_{T_{CO_2}}$ depends on the number of gas molecules per unit time, t, leaving the muscle tissue and entering the tracheal system (dN_T), and the outflow of gas molecules through the open spiracles (dN_A). This relationship can be expressed as:

$$P_{T_{CO_2}}(t) = P_{T_{CO_2}}(t-1) + kTV_T^{-1}[dN_T(t) - dN_A(t)] , \quad (1)$$

in which k is the Boltzmann constant, T is temperature, and V_T is tracheal volume. For convenience we have set kTV_T^{-1} equal to 1.0. According to diffusive theory, which defines mass flow rate into and out of the tracheal system as the product between gas conductance and partial pressure difference, the changes in the quantities dN_T and dN_A are given by:

$$dN_T = (P_{M_{CO_2}} - P_{T_{CO_2}})G_c dt \text{ and } dN_A = (P_{T_{CO_2}} - P_{A_{CO_2}})G_s dt , \quad (2)$$

respectively (Kestler, 1985). In this equation partial pressure of CO_2 in the muscle is $P_{M_{CO_2}}$ and in the ambient air is $P_{A_{CO_2}}$, the term G_c is gas conductance of the cytoplasm and G_s is instantaneous gas conductance of the spiracle opening for CO_2.

Spiracle function was modeled on the base of two previous

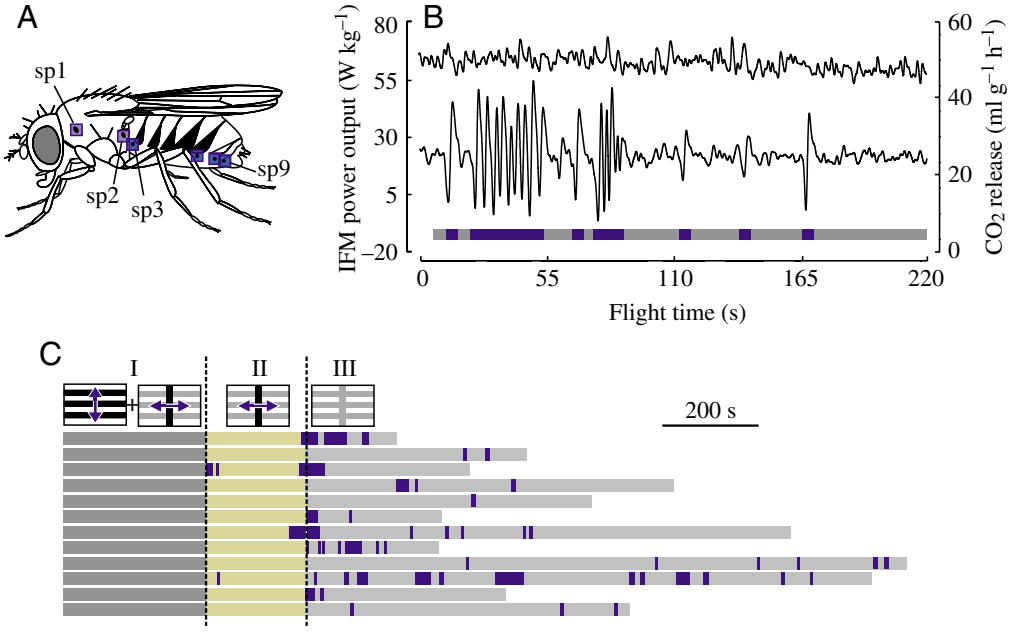

Fig. 1. Oscillatory release of CO_2 during tethered flight in a single fruit fly *Drosophila*, flying in a flow-through respirometic chamber of a virtual-reality flight arena and exhibiting only small fluctuations in metabolic rate. (A) Location of spiracle openings on one side of *Drosophila*: sp1, mesothoracic spiracle; sp2, metathoracic spiracle; sp3–9, abdominal spiracles. (B) Muscle mass-specific mechanical power output of the asynchronous indirect flight muscles (IFM) *in vivo* was calculated from simultaneous measurements of aerodynamic flight force production, wing stroke amplitude and frequency (left scale, top trace). Bottom trace shows muscle mass-specific CO_2 release rate (right scale). In the bar below, blue indicates the oscillatory phase of gas release; gray, non-cyclic gas release. (C) Temporal distributions of oscillatory CO_2 release patterns (blue) of 12 fruit flies flown under three different experimental conditions (shown in the pictograms): (I) fruit flies vary metabolic rate in response to visual stimulation by external open-loop vertical motion of horizontal stripe patterns while simultaneously themselves stabilizing the azimuth position of a closed-loop vertical stripe using the relative difference in wing stroke amplitude (dark gray, left; see Materials and methods); (II) flight under visual-closed-loop conditions but in the absence of lift stimuli (yellow, middle); and (III) flight in absence of any moving visual objects (gray, right). Data show that oscillatory releases of CO_2 occur randomly and are essentially restricted to flight sequences without any visual stimulation of the surrounding panorama (stationary patterns).

findings in flying *Drosophila*. (1) Optical recordings of the spiracle opening area have shown erratic opening activity during flight, suggesting that spiracles control gas flux by continuously varying their opening area. (2) When the fly varied metabolic rate between minimum and maximum values, tracheal partial pressure for CO_2 and oxygen remained essentially unchanged (Lehmann, 2001). The latter finding is also supported by direct measurements of muscular partial pressure of oxygen in resting and flying moths and honey bees. In both insects, muscular partial pressure of oxygen during flight remains close to resting values (approximately 8.57 kPa in the moth and 6.36 kPa in the honeybee; Komai, 1998, 2001). According to these data, we employed a binary function that models spiracle conductance for two separate states: below (spiracle lids are predominantly closed) and above (lids are predominantly open) a tracheal partial pressure threshold value T_s. During oscillatory gas release, the 'predominantly closed' state allows tracheal partial pressure to rise over time, whereas the 'predominantly open' state removes CO_2 out of the tracheal system faster than the flight muscles deliver the gas, resulting in a decrease of tracheal partial pressure. This simple relationship can be expressed as:

$$G_S = rnd(0\ldots0.5)G_{S,max}\text{ , for } Ts \geqslant P_{TCO_2}(t-\delta)\text{ ,} \quad (3)$$

and as:

$$G_S = rnd(05.\ldots1.0)G_{S,max}\text{ , for } Ts < P_{TCO_2}(t-\delta)\text{ ,} \quad (4)$$

in which $G_{S,max}$ is maximum spiracle opening area, *rnd* is an equally distributed random function and δ is the response time of the model spiracle to changes in tracheal gas concentration (spiracle hysteresis). The response time determines solely the frequency of cyclic gas release rate and does not change any other property of the analytical model. Since δ should be larger than the simulated time interval dt, that is 0.25×10^{-3}, we have arbitrarily set δ to 45dt. At P_{ACO_2}=0, as used in our flight arena experiments, and assuming that all four thoracic spiracle contribute equally to CO_2 gas release, total CO_2 release rate $\dot{M}_{CO_2,tot}$ is eventually given by:

$$\dot{M}_{CO_2,tot}(t) = \sum_{i=1}^{4} P_{TCO_2,i}(t)G_{S,i}(t)\text{ .} \quad (5)$$

Eventually, model data and respirometric measurements obtained in the flying fruit fly were smoothed equally using a 20 data points running average filter in Origin 7.0 (Microcal).

Results

Respirometric measurements

Fig. 1B shows a typical flight sequence of a tethered flying fruit fly in which the animal exhibits both non-cyclic gas release (gray) and periodical oscillation of CO_2 release (blue), while producing only small-scale fluctuations in flight-muscle specific mechanical power output. The data apparently show that gas release oscillations occur randomly in a flight sequence and often in bursts of up to 18 cycles in a row (Figs 1C, 2A,B). The frequency of the respiratory cycles is approximately 0.37±0.055 Hz (mean ± S.D., 180 cycles, N=12 flies; for single flies, mean S.D. = 0.097 Hz; Fig. 2C,D). On average, gas release rate during oscillatory breathing behavior is not significantly different from flight sequences yielding non-oscillatory CO_2 release patterns (t-test, P>0.05, 'cyclic' CO_2 release = 26.5±1.5 ml g^{-1} flight muscle h^{-1}, 'non-cyclic' = 26.6±1.2 ml g^{-1} h^{-1}, N=19 flight sequences, 401 s total flight time), suggesting that oscillatory breathing does not result from a pay-off of anaerobic debt during elevated locomotor activity. Moreover, Fig. 1C shows that oscillatory gas exchange patterns in *Drosophila* mostly occur in flight sequences without visual stimulation (section III, Fig. 1C), during which the temporal changes in metabolic rate are negligible and total flight force remains close to hovering force production (flight force/body weight = 0.93±0.20, body mass = 0.99±0.22 mg, means ± S.D., N=8 out of 12 flies; Fig. 1C, sections II and III). We rarely observed oscillatory gas release patterns near maximum

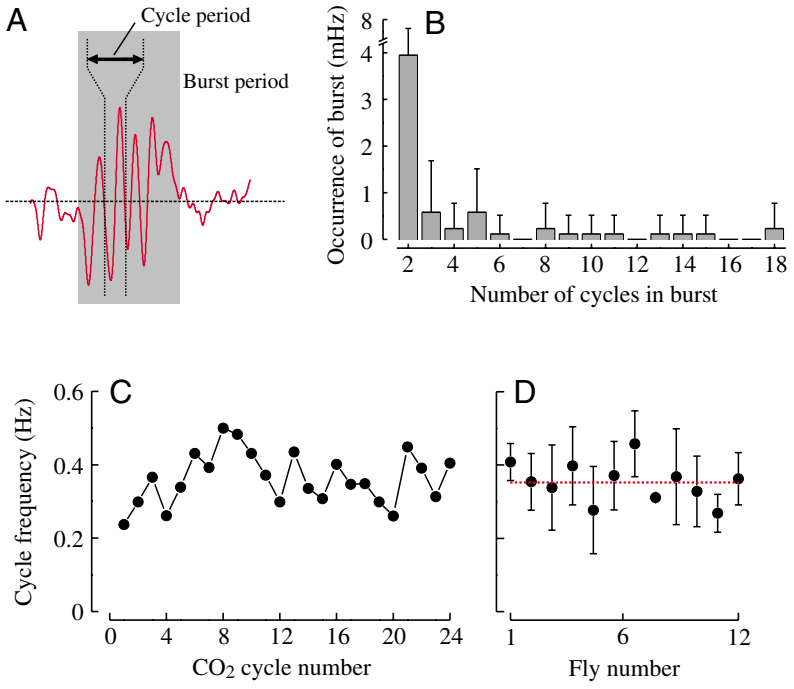

Fig. 2. Temporal occurrence and frequency of cyclic gas exchange patterns in flying *Drosophila*. (A) Cyclic gas release often consists of multiple consecutive CO_2 release cycles in a burst (gray). (B) Frequency of occurrence of cyclic bursts containing different numbers of cyclic CO_2 'waves'. N=12 flight sequences, as shown in Fig. 1C (12 flies). (C) Frequency of consecutive CO_2 cycles in a single fly. (D) Mean frequency of CO_2 oscillations measured in 12 flies. Red dotted line, mean value derived from all 12 flies. Values are means ± S.D.

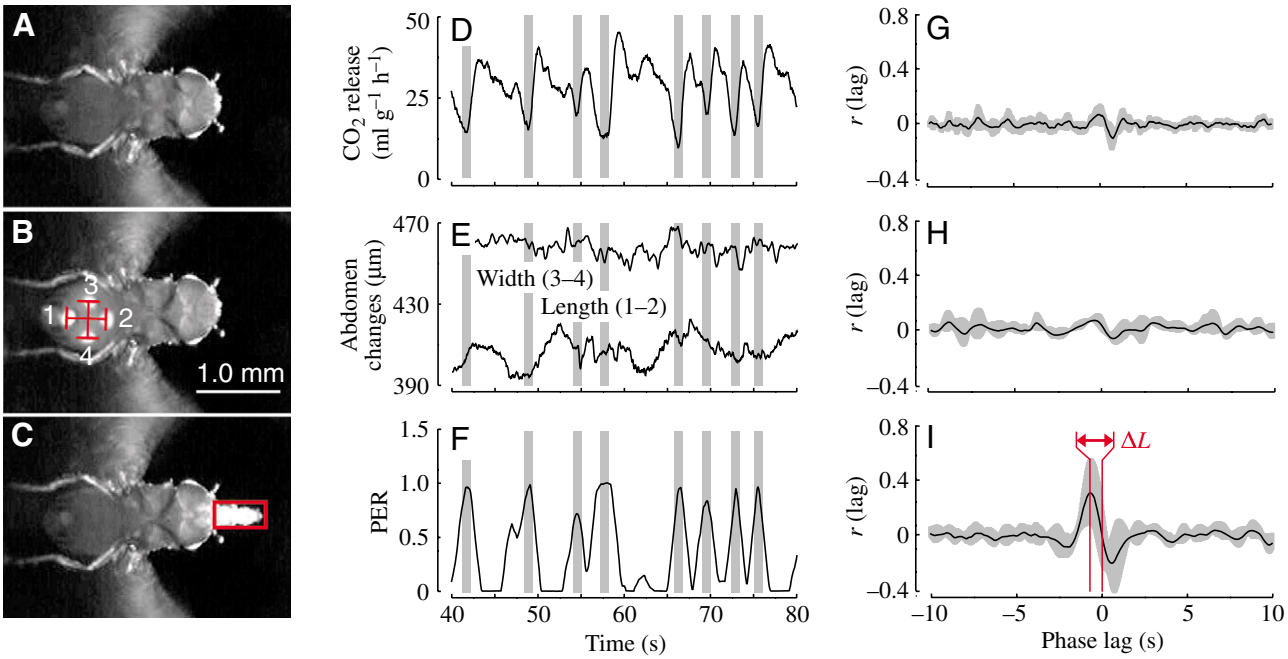

Fig. 3. Potential mechanisms of oscillatory CO_2 release patterns in *Drosophila*. (A–C) Infra-red video images show the flying fly from below, while recording wing kinematics, flight force production and the release of flight muscle-specific CO_2. Four UV light-activated fluorescent markers on the animal's abdomen (B) allow video-based in-flight tracking of abdominal pumping movements, and light intensity changes within the measurement area (red box) indicate proboscis movements during flight (C). (D–F) Simultaneously recorded flight data of (D) CO_2 release, (E) abdominal length and width changes based on movement of markers in B and (F) occurrence of the proboscis extension reflex (PER), during a 40 s flight sequence. A PER value of zero indicates that the proboscis is fully retracted, whereas a value of 1.0 means full extension. Gray bars indicate examples where CO_2 release decreases (inhalation) as the fly extends the proboscis. No moving visual stimuli were displayed in the surrounding panorama. (G–I) Cross-correlation coefficients r are plotted between (G) the derivative of muscle mass-specific mechanical power output of the flight muscles and the derivative of CO_2 release, (H) the derivative in abdominal length and CO_2 release, and (I) the derivative of proboscis movements and CO_2 release. ΔL = cross-correlation temporal phase shift between data sets (phase lag). Each cross-correlation analysis was performed for six flight sequences over time t, using a sliding data window with $0.5t$ width. Length of the flight sequences was 141±77 s (mean ± S.D., N=3 flies). In this analysis we limited our data set to flies that showed pronounced and long-lasting gas release oscillations. Mean correlation coefficient r is plotted in black; gray areas indicate S.D.

locomotor capacity of approximately 1.6 times the hovering flight force, suggesting that cyclic breathing is not needed to satisfy oxygen demands at maximum metabolic rate. Experiments in which we changed the order of the presented stimulus conditions revealed that cyclic gas release does not occur as a consequence of previously applied open- or closed-loop feedback conditions. To validate the relationship between locomotor output of the animal and gas release statistically, we calculated the temporal cross-correlation coefficient r between flight muscle mass-specific mechanical power output and CO_2 release rate (Fig. 3A,D,G). Superficially, the small coefficients show no preferred phase lag between both parameters, implying that none of the temporal fluctuations during CO_2 release oscillations are correlated with the animal's changes in metabolic rate (maximum r=0.07 at phase lag = –0.08s).

Video analysis

To tackle the significance of abdominal pumping as a source of the measured CO_2 fluctuations in *Drosophila*, we monitored the geometry (length and width) of the abdomen during flight using an automatic video tracking technique (Fig. 3B). Although the data that have been reconstructed from single video images show systemic changes in abdominal geometry, the changes were quite small and consistently below approximately 100 µm (Fig. 3E). The temporal cross-correlation coefficient r, averaged over six flight sequences all exhibiting pronounced and long-lasting gas release oscillations (sequence length = 141±77 s, mean ± S.D.), shows that superficially none of the abdominal length changes appear to be correlated with the cyclic changes in gas exchange rate (maximum r=0.06 at phase lag = –0.44 s, Fig. 3H).

As a second explanation for our experimental data, we considered whether cyclic gas exchange is due to changes in haemolymph pressure caused by any ventilatory function of the proboscis. In *Drosophila*, the proboscis is relatively large and its volume amounts to approximately 15–20% of the head's volume (~0.2 mm^3; Demerec, 1965). Fruit flies regularly extend and retract their proboscis during flight, a behavior that might enlarge (inhalation) or compress (exhalation) the large paired frontal, postgenal and postocular air sacs in the fly's head (Lehmann et al., 2000). We quantified voluntary proboscis movements by mapping light intensity changes of

the video images in a defined region shown by the red box in Fig. 3C. Cross-correlation analysis between the derivative of proboscis motion and CO_2 release shows the following. (1) In single flies, up to 80% of the variance in tracheal CO_2 release fluctuations can be assigned to PER activity. The mean correlation coefficient amounts to 0.31±0.24 (mean ± S.D., N=6 sequences, 3 flies, Fig. 3I), which is at least fivefold higher than the maximum temporal correlation coefficient between CO_2 release and abdominal movements (0.31 *vs* 0.06). (2) There is a small temporal shift of the maximum cross correlation value with respect to zero phase lag, indicating that the proboscis starts moving approximately 0.6 s before the fly changes its gas release rate (ΔL, Fig. 3I).

Spiracle modeling

Besides the employment of convective strategies for breathing, oscillatory gas release in diffusion based respiratory systems may also result from changes in spiracle opening area, similar to the mechanism causing the discontinuous gas exchange cycle in a resting insect (Lighton, 1994; Miller, 1981; Slama, 1994; Snyder et al., 1995). In this scenario, the proboscis extension reflex (PER) would only be correlated with CO_2 release rather than being the primary cause for its oscillatory behavior. In insects, spiracle muscle control is CNS-mediated but the muscle activity also depends on local concentrations of respiratory gases (for a review, see Nikam and Khole, 1989). In the locust *Schistocerca gregaria*, for example, the mesothoracic closer muscle is innervated by two excitatory motorneurons and a peripherally located neurosecretory cell (Swales et al., 1992). Due to their small size it is difficult to record electrically from spiracle muscles in flying *Drosophila*. The same holds for direct measurements of total spiracle opening area, because the mesothoracic spiracle is partly covered during flight by the beating haltere. For this reason, we cannot reject *per se* the hypothesis that the CNS synchronously opens and closes multiple thoracic spiracles *via* excitatory motorneurons.

However, even assuming that no CNS activity is involved in spiracle control and that each spiracle functions autonomously, gas release may start to oscillate in cases where several spiracles stochastically synchronize their opening activities. In flying *Drosophila*, total spiracle opening area matches the respiratory exchange area of the diffusive path exactly to the actual needs, and thus tracheal partial pressures for CO_2 and oxygen are stabilized in a narrow range between 1.3–1.4 kPa and 19.8–19.9 kPa, respectively (Lehmann,

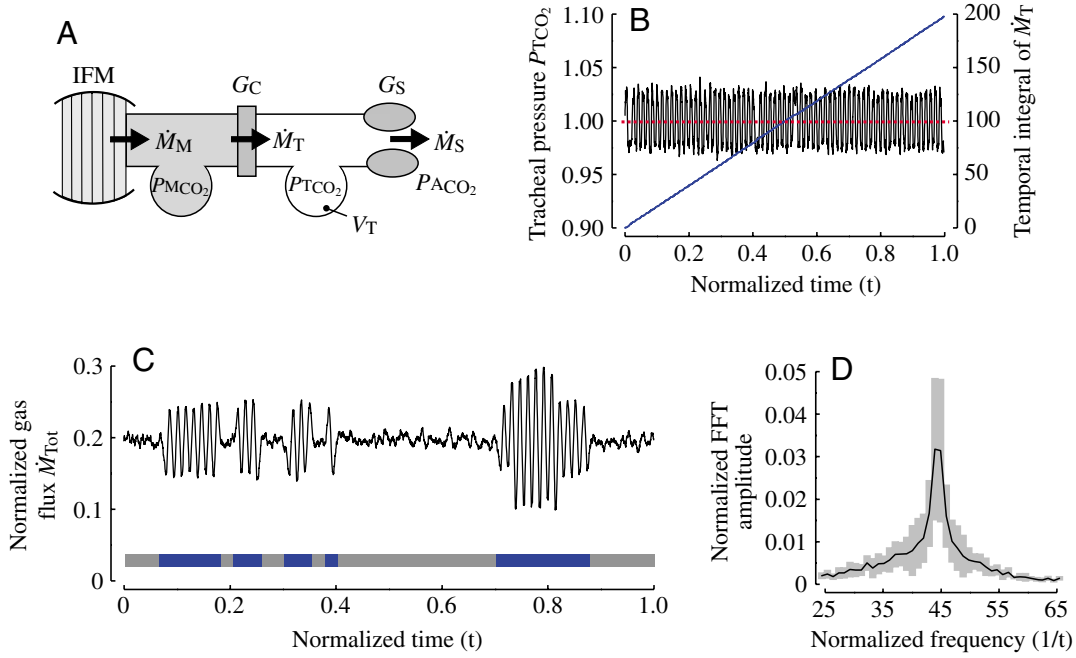

Fig. 4. Analytical modeling of spiracle function. (A) Schematics of the diffusive model, as used in the present study. CO_2 flux into and out of the tracheal system depends on the pressure difference (P_{MCO_2}–P_{TCO_2} and P_{TCO_2}–P_{ACO_2}) multiplied by the conductance for CO_2 through the cytoplasm and the spiracle opening, G_C and G_S, respectively. IFM, indirect flight muscle; $\dot{M}_M$, metabolic rate of the flight muscle; $\dot{M}_T$, temporal flux of CO_2 molecules entering the tracheoles of the tracheal system; $\dot{M}_S$, gas flux through the spiracle. More details are given in the Materials and methods. (B) Example of simulated instantaneous tracheal partial pressure of CO_2 as controlled by a single model spiracle. Switching opening behavior of the model spiracle stabilizes P_{TCO_2} near a threshold value T_s (red, left scale). Temporal sum of $\dot{M}_T$ is shown in blue (right scale). (C) Example of simulated total release rate of CO_2 of four autonomously working model spiracles, as shown in B. Due to temporal beat, the four modeled spiracle openings may synchronize (oscillatory gas release, blue) or may work out of phase (non-oscillatory release, gray). (D) Relative amplitude of Fast-Fourier Transformation (FFT) analysis of simulated data traces. Location of peak (black) indicates the principle frequency component of the FFT spectrum. Model parameters are: T_s=1.0, G_c=1.0, $G_{S,max}$=0.1, P_{MCO_2}=1.05. Gray area in D shows S.D. of mean value (black) obtained from 20 different randomly distributed starting values for P_{TCO_2}. t=total length of normalized time domain (0–1).

2001). It seems thus likely that spiracles open and close around a threshold value for tracheal partial gas pressures. Inspired by these observations, we analytically modeled gas release for each of *Drosophila*'s four large thoracic spiracles, employing a simple diffusive model for the control of tracheal CO_2 partial pressure (see Materials and methods, Fig. 4A). Using the 'fruit fly-inspired' characteristics of spiracle function, and depending on the parameter settings, each model spiracle is able to produce small-scale fluctuations in tracheal CO_2 partial pressure at constant metabolic rate autonomously. Consequently the rate at which muscular CO_2 enters the distal endings of the model tracheae (tracheoles) changes only slightly (slope of blue line, Fig. 4B). As long as the four model spiracles open and close out of phase, the total release rate of CO_2 is non-cyclic (gray, Fig. 4C). If certain conditions are met, however, opening activity randomly synchronizes, producing large oscillations of gas release similar to the pattern observed in the flying fruit fly (blue, Figs 1B, 4C). This result is independent of the initial tracheal partial pressure ($P_{T_{CO_2}}$) for the simulation that was shown by selecting 20 different randomly distributed starting values for $P_{T_{CO_2}}$ (Fig. 4D).

Multiple variations of the analytical model parameters show that the likelihood of the occurrence of oscillatory gas release depends at least on two parameters: (i) the ratio between muscular partial pressure and the spiracle threshold values for that gas (Fig. 5A,B) and (ii) the ratio between maximum conductance of each model spiracle and the simulated conductance of the cytoplasm between muscle tissue and the tracheoles (Fig. 5C,D). The first prerequisite implies that cyclic CO_2 release only occurs when the tracheal partial pressure for CO_2 is allowed to increase when spiracles are held predominantly closed, and to decrease when the spiracles are predominantly open over time. The analytical model thus predicts that oscillatory release patterns disappear when tracheal partial pressure for CO_2 is consistently below or above the spiracle threshold value for this tracheal gas (black traces, Fig. 5B). At high metabolic rates, for example, all four model spiracles stay predominantly open (between 0.5 and $1.0G_{S,max}$) because the tracheal partial pressure of the respiratory gas remains above the opening threshold value ($P_{M_{CO_2}}/T_s \geqslant 1.1$, Fig. 5A,B). Under these conditions, any changes in gas release rates reflect changes in metabolic rate or differences in partial pressure between the tracheal system and the ambient air, but not changes in the spiracle's own diffusive exchange area. In contrast, the changes in gas release pattern are more subtle when changing the ratio between cytoplasm and maximum spiracle conductance (Fig. 5C). At the given parameter settings, small ratios below 0.1 suppress the likelihood of cyclic release patterns because CO_2 gets completely removed out of the tracheal system even at the leaky ('predominantly closed') spiracle state. In contrast, higher conductance ratios between 0.2 and 0.8 consistently produce pronounced temporal fluctuation in gas release rate. With increasing $G_{S,max}/G_c$ ratio >0.1, however, the amplitude of the data's main FFT frequency component (i.e. equal to spiracle hysteresis δ) simultaneously decreases, and the broader distribution of frequency components in the time domain indicates that the gas release rates become increasingly noisy (Fig. 5D).

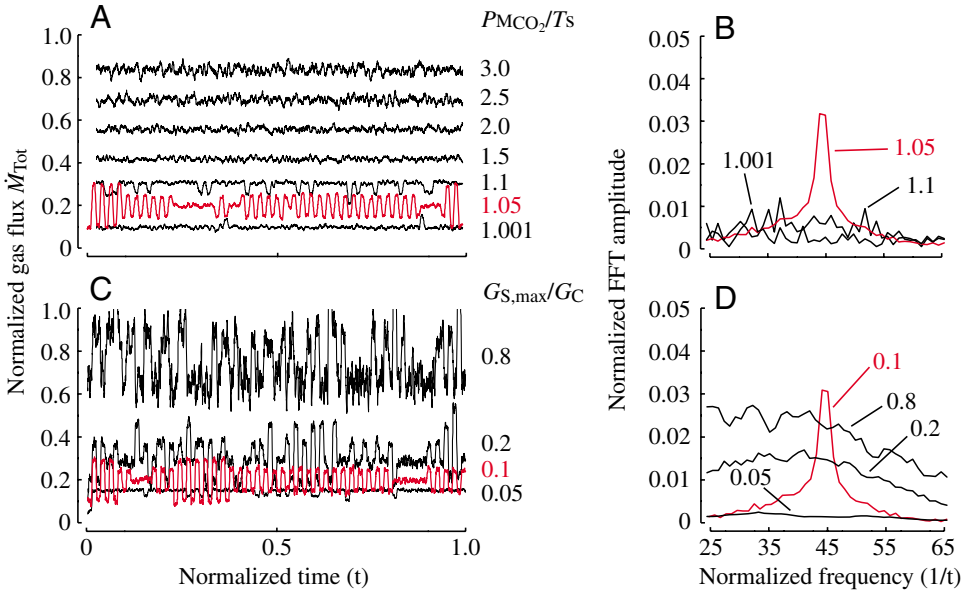

Fig. 5. Total CO_2 gas release rate through 4 autonomously working spiracles, modeled by a simple analytical approach for tracheal gas exchange. In all simulations spiracle threshold value for opening T_s was set to 1.0. (A) The likelihood of cycling gas release due to synchronization of spiracle opening activity depends on the ratio between the modeled muscular partial pressure of the gas $P_{M_{CO_2}}$ and the spiracle threshold value T_s. At model parameters of G_c=1.0 and $G_{S,max}$=0.1, ratios around 1.05 produce gas release traces similar to the release pattern produced by the flying fly. (B) Relative amplitude of Fast-Fourier Transformation (FFT) analysis of the simulated traces shown in A. The main frequency component and thus cyclic response of the model disappears when $P_{M_{CO_2}}/T_s$ ratio is below or above approximately 1.05 (red). Traces represent mean values of ten simulated model runs that have been smoothed using a 3-point running average, respectively. (C) Changes in CO_2 release pattern in response to variations of maximum spiracle opening conductance ($G_{S,max}$) expressed as the ratio between $G_{S,max}$ and CO_2 conductance of the cytoplasm (G_c). At a parameter setting of T_s=1.0, G_c=1.0 and $P_{M_{CO_2}}$=1.05, cyclic release patterns disappear at ratio below approximately 0.1 but persist at higher ratios. (D) FFT amplitude spectrum of the model traces shown in C. For more information see explanation in B.

Discussion

PER induced ventilation

The data presented in the present study suggest that cyclic gas release patterns during constant locomotor activity in flying *Drosophila* might result from at least three distinct respiratory mechanisms: (i) ventilation caused by the proboscis extension reflex, (ii) synchronized spiracle activity mediated by the CNS which we could not validate experimentally, and (iii) stochastic synchronization of autonomously working spiracles, as suggested by analytical modeling. Although PER ventilation and spiracle synchronization behavior might result in similar breathing patterns, their functional relevance for the animal is quite different. PER ventilation produces convective flow and should thus increase (decrease) tracheal partial pressure of oxygen (CO_2) in the flying fruit fly, whereas as spiracle synchronization behavior should not affect local partial pressure for tracheal gases. PER mediated ventilation thus compares to the most common mechanism for ventilation in insects, i.e. abdominal pumping (for a review, see Wigglesworth, 1972). In contrast to other insects, however, the small cross-correlation coefficients below approximately 0.06 (Fig. 3H) suggest that abdominal pumping is not the primary source for cyclic gas release patterns in flying *Drosophila*. Moreover, it is also unlikely that mechanical deformations of the thoracic box (auto-ventilation) cause cyclic breathing patterns, as mentioned in the Introduction, for two reasons: firstly, the length changes of the thorax between upstroke and downstroke of the wings in *Drosophila* are relatively small and amount to not more than approximately 2% of the overall thoracic length (Chan and Dickinson, 1996). Secondly, wing stroke frequency in *Drosophila* is approximately 200 Hz and thus 540 times higher than CO_2 cycling frequency of approximately 0.37 Hz (Lehmann and Dickinson, 1998). Instead, the high cross-correlation coefficient between tracheal CO_2 release fluctuations and PER in conjunction with the temporal timing between both events (0.6 s phase advance of PER) strongly suggests a functional relevance of PER for respiratory gas exchange. This view on respiration in the fruit fly is also supported by the finding that the variance of PER frequency in single flies and among different flies is notably small (single fly = 2.6% of 0.37 Hz, multiple flies = 0.8% of 0.37 Hz), suggesting an internal neuronal pacemaker for the PER breathing rhythm rather than random activity of the proboscis' retractor and extensor muscles. Alternatively, we should also take into consideration that PER-mediated convection simply occurs as a by-product of proboscis extensions, serving a different yet unknown task during flight. In conclusion, our analysis offers a novel and quite unconventional mechanism for reinforcing respiratory gas exchange rates in an insect. Considering the similarities in morphology, proboscis-induced ventilation might become increasingly important for larger flies that rely on convective flow into and out of their tracheal system during elevated locomotor performance.

Modeling synchronized behavior of spiracle muscles

In the past, several researchers have attempted to model respiratory processes. At the single cell level, Thumfort et al. (2000) recently modeled oxygen diffusion by computer simulation in three dimensions and applied this model to a case study. They found that generating a one-dimensional representation of the three-dimensional surface of the cell is a close approximation to the more complex three-dimensional model with systematic differences below 10%. Research on diffusive models of the entire tracheal system of insects was pioneered by a study of Weis-Fogh (1964), who developed an extensive set of equations for steady state diffusion in an isotropic tissue, tracheal gas transport and exchange (air–tube diffusion). Weis-Fogh, for example, also considered different topologies of diffusive systems and estimated their effect on gas exchange. Later, Kestler (1985) also modified these models towards ventilation and focused on different gas exchange models describing respiratory flux between the tracheoles and mitochondria. Lehmann (2001) applied Kestler's model to diffusive gas exchange and respiratory water loss through the spiracle in tethered flying *Drosophila* and demonstrated how the water loss rate can be used to derive total spiracle opening area in the animal *in vivo*. Snyder et al. (1995) proposed an elaborate model for cyclic ventilation in insects that also covers the three phases of cyclic ventilation. The authors basically reported that volume expansion of the trachea, and not an increased cross-sectional area of the spiracles *per se*, is the important adaptation to normobaric hypoxia. However, although all elegant, none of the elaborate studies above have considered the potential complex temporal interactions in diffusive gas exchange between multiple spiracles.

The simplified analytical model of tracheal diffusion presented here offers an alternative explanation for the experimental data achieved in the flying fruit fly. The model proposes that the temporal switching between non-cyclic and cyclic breathing patterns can be explained by phase transitions between (i) times during which opening activity of the four autonomously working thoracic spiracles in the fly synchronize (cyclic breathing) and (ii) times at which the spiracle-controlled total diffusive areas are temporally out-of-phase (non-cyclic breathing). Interestingly, recent data on respiration in resting ants *Camponotus* seem to support this scenario also occurring during the discontinuous gas exchange cycle (Lipp et al., 2005). Multiple studies have shown that resting ants typically release a single peak of CO_2 during the DGC's opening phase ('O'-phase; Lighton, 1992, 1994, 1996; Lighton and Feener, 1989), which is consistent with the idea of synchronized spiracle opening activity, assuming that multiple spiracles participate in gas exchange. In comparison, the recent study on *Camponotus* gas release measured with high-temporal resolution showed that resting animals also release CO_2 as multiple 'O'-peaks within a single DGC cycle (Lipp et al., 2005). One explanation for the latter finding could be that it is a consequence of desynchronized opening activity of at least two spiracle muscles, which compares to the non-cyclic gas

release pattern in our flying insect. The temporal distribution of the gray areas in Fig. 1B,C shows that in flying *Drosophila*, the desynchronized spiracle opening condition seems to be more common and, in addition, not all tested flies have shown cyclic breathing patterns. In most recordings of flying fruit flies, instantaneous gas exchange rate thus matches the actual metabolic needs of the animal, as reported previously (Lehmann, 2001).

One of the most interesting predictions of our analytical model is that cyclic CO_2 release patterns do not necessarily require large changes in diffusive area of a single spiracle during control behavior, as indicated by the small (±3%) tracheal partial pressure changes in the example shown in Fig. 4B. Under certain conditions, these small changes in diffusive area, in conjunction with concomitant small changes in tracheal partial pressure, appear to be sufficient to modulate total CO_2 release rates of up to ±50% peak-to-peak of the mean value, when all four model spiracles open and close at similar phase, as shown in Fig. 4C. The most critical prerequisite for the occurrence of CO_2 release cycling of the analytical model seems to be that tracheal pressure of CO_2 decreases (increases) when the model spiracle opens between 50% and 100% (0 and 50%) of the maximum diffusive area, as mentioned above. If metabolic rate causes tracheal partial pressure for CO_2 to increase above the spiracle opening threshold (T_s), cyclic respiration vanishes and the modeled instantaneous CO_2 release rate matches instantaneous metabolic activity. This observation could potentially explain why, in the experiments performed with flying *Drosophila*, oscillatory gas release patterns only occurred at flight forces and metabolic rates well below maximum locomotor capacity.

Due to the lack of elaborate data for *Drosophila*'s respiratory system, including muscular partial pressure estimates, gas conductance of the cytoplasm and flow conditions inside the tracheae, it is difficult to model gas release using exact physiological values (Kestler, 1985). The proposed analytical model, including all parameter settings, should thus be considered as a rough hypothesis on how tracheal gas release can potentially be shaped by stochastic spiracle opening and closing processes. Moreover, our simplified analytical model makes some inherent assumptions about spiracle control strategies of the living organism (e.g. binary random function for spiracle opening/closing behavior). The results derived from the analytical model, including any comparison with data recorded in the flying animal, should thus be treated with caution. Nevertheless, considering all limitations and problems of our simplified analytical model for insect respiration, the coincidence between the data produced by the simulation and the flying fly is marked, and thus might highlight a fundamental inherent property of spiracle function in diffusion-based tracheal systems of small insects.

Conclusions

In sum, this study proposes that periodically oscillating gas release patterns in flying *Drosophila* might result from at least two unconventional respiratory mechanisms: firstly, the proboscis appears to serve as a pumping organ for ventilation, and secondly, gas release oscillations may come about by synchronized opening activity of the large thoracic spiracles similar to the DGC. Interestingly, in the fruit fly both mechanisms are thought to have little significance for flight muscle function, and ventilation is apparently not required to satisfy the high oxygen demands even at maximum locomotor capacity (Weis-Fogh, 1964; Lehmann, 2001). Instead, a possible advantage of the proboscis extension reflex for tracheal ventilation might be to actively promote local oxygen supply of the animal's head. In dipteran flies, the retina and optic lobes may require at least 20% of the resting metabolic rate due to the highly specialized and large visual system of flies (Laughlin, 1987). Since there are no spiracles in the head, respiratory gases must pass through small tracheae inside the approximately 80 μm diameter neck connective (Demerec, 1965). If correct, the hypothetical benefit of the proboscis-induced ventilation for breathing might be to circumvent this bottleneck for diffusive respiration, in order to ensure evacuation of CO_2 from – and the supply of oxygen to – the fly's brain. Eventually, this behavior might balance tracheal partial pressures of respiratory gases locally within the body compartments of *Drosophila* during certain flight conditions.

List of symbols and abbreviations

DGC	discontinuous gas exchange cycle
dN_A	outflow of gas molecules through the open spiracles
dN_T	number of gas molecules entering the tracheal system from the muscle
IFM	indirect flight muscle
G_c	gas conductance of the cytoplasm
G_S	instantaneous gas conductance of the spiracle opening
$G_{S,max}$	maximum spiracle opening area
k	Boltzmann constant
$\dot{M}_{CO_2,tot}$	total CO_2 release rate
$\dot{M}_M$	metabolic rate of the flight muscle
$\dot{M}_S$	gas flux through the spiracle
$\dot{M}_T$	temporal flux of CO_2 molecules entering the tracheoles of the tracheal system
$P_{A_{CO_2}}$	partial pressure of CO_2 in ambient air
PER	proboscis extension reflex
$P_{M_{CO_2}}$	partial pressure of CO_2 in the muscle
$P_{T_{CO_2}}$	partial pressure of CO_2 in the trachea
r	cross-correlation coefficient
rnd	equally distributed random function
SNR	signal-to-noise ratio
T	temperature
t	normalized time
t	time
T_s	tracheal partial pressure threshold value
V_T	tracheal volume

ΔL cross-correlation temporal phase shift between data sets

δ response time of the model spiracle to changes in tracheal gas concentration (spiracle hysteresis)

τ time constant

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