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RESEARCH ARTICLE

Periodic heartbeat reversals cause cardiogenic inspiration and expiration with coupled spiracle leakage in resting blowflies, *Calliphora vicina*

Lutz T. Wasserthal*

ABSTRACT

Respiration in insects is thought to be independent of the circulatory system because insects typically lack respiratory pigments and because oxygen transport occurs in the gaseous phase through a ramified tracheal system by diffusion and convection directly to the tissues. In the blowfly, as in other insects with periodic heartbeat reversal, the haemolymph is periodically shifted between the anterior body and abdomen, exerting alternating pressure changes on the compliant tracheae in the thorax and in the abdomen. Simultaneous pressure and O_2 optode measurements show that, during negative pressure periods, the tracheal partial pressure of oxygen (P_{O_2}) increases by 0.5 kPa. In the quiescent fly, tracheal P_{O_2} is rather high (17.5–18.9 kPa), although the thoracic spiracles remain constricted. Microscopic video recordings and reflectance measurements revealed that the dorsal soft edges of the valve lips of the second spiracle leave a very small leak, which is passively widened during backward pulses of the heart. Thus, negative pressure, combined with increased leakage of the spiracle Sp2 valve enable inspiration in the thorax. The positive pressure periods are correlated with a new type of convective CO_2 micro-bursts as shown in flow-through measurements. The bulk of the CO_2 is, however, released after longer interbursts in macro-bursts with actively opening valves reminiscent of the open phase in a cyclic gas exchange. When the valves open, the P_{O_2} in the thoracic air sacs unexpectedly drops by a mean of 2.75±1.09 kPa, suggesting a displacement of O_2 by the transient accumulation of CO_2 in the tracheal system before its release.

KEY WORDS: Insect respiration, Circulation, Tracheal pressure, Tracheae, Spiracles, Oxygen, Haemolymph, Cyclic gas exchange, Suction ventilation, CO_2 release, Macro-burst, Flutter phase, Water retention

INTRODUCTION

Insects breathe by a branching air tube system called the tracheae, formed by lateral segmental invaginations of the exoskeleton. The blind tracheolar ends invade the tissues, supplying them directly with oxygen. In many flight-adapted adult insects, the tracheae are partly enlarged to form air sacs, which are suspended between the exoskeleton and the organs and occupy a vast part of the body cavity. The inflow and outflow of respiratory air is regulated by valves behind the filter-protected atrium of the lateral openings, called the spiracles. The gas exchange in many resting insects is characterized by cyclic CO_2 release. These widely distributed cyclic mechanisms have been studied for many years (Schneiderman, 1960; Lighton, 1996; Marais et al., 2005; Chown et al., 2006). The majority of recent investigations focussed on the impact of physical ecological factors, such as temperature, humidity and gas concentrations, on the diverse gas exchange patterns to evaluate their functional significance and ecological or evolutionary advantages.

The aim of this study was to analyse the assumed functional interplay of the periodic heartbeat reversals with the respiratory cycles and the involved structures such as the heart and spiracular valves. Their action in quiescent Diptera has scarcely been visualized or recorded, because direct observation of most valves is difficult. They are hidden behind dense filter structures composed of ramified trichomes. Therefore the influence of the valves on gas exchange was deduced from the CO_2 output measured by flow-through experiments (Kestler, 1985; Lighton, 1988; Hetz et al., 1994), partly with cannulation to specified spiracles or compartments of the body (Wasserthal, 2001; Duncan and Byrne, 2002), by thermographic-anemometric (Wasserthal, 1981; Slama, 1988) and volumetric-manometric measurements of expired or consumed air (Jõgar et al., 2007; Karise et al., 2010; Jõgar et al., 2011). A more immediate influence of the spiracles on the gas exchange was revealed by intra-tracheal pressure recordings (Brockway and Schneiderman, 1967; Kestler, 1985; Hetz et al., 1994) and oxygen uptake (Hetz et al., 1994; Wobschall and Hetz, 2004; Matthews and White, 2011). The gas exchange cycles are often characterised by a sequence of constriction, fluttering and opening of the spiracles (CFO) without or with coordinated bouts of ventilation movements (CFV) (Kestler, 1985), and are called discontinuous or in the absence of constriction cyclic gas exchange (DGC or CGE) (Lighton, 1996). It has been shown in *Calliphora* and *Drosophila* that periodic heartbeat reversal causes changes in haemocoelic and tracheal pressure and volume, alternating in the anterior body and in the abdomen (Wasserthal et al., 2006; Wasserthal, 2007; Wasserthal, 2012) because the tracheal systems and haemocoels of both compartments are functionally separate (Faucheux, 1973; Wasserthal, 1999). Therefore, it was hypothesized that the haemolymph shift ventilates the tracheal system by alternating between the anterior and posterior body. Under resting conditions, the tracheal pressure differs from the atmosphere because the spiracles impede immediate gas exchange and pressure equalization with the atmosphere by the more-or-less closed valves.

The action of the thoracic spiracular valves was analysed by pressure measurements directly at the intact spiracles. It was of interest to see whether the valves open and close synchronously or in a different manner. Simultaneous measurements at two spiracles was necessary to determine whether observation of one spiracle is representative for the other spiracles of the thorax. In order to avoid

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List of abbreviations

CFO	constricted–flutter–open
CFV	constricted–flutter–ventilation
CGE	cyclic gas exchange
DGC	discontinuous gas exchange cycle
PSV	passive suction ventilation
Sp1	mesothoracic spiracle
Sp2	metathoracic spiracle

a blockage of the gas exchange by the tight sensor attachment directly at the spiracles, most pressure measurements and all oxygen measurements were performed by intubating one of the dorsal air sacs, which proved to be a mildly invasive procedure. For analysis of the influence of the spiracular valves on the uptake of oxygen and the release of the CO_2, simultaneous video recordings and reflectance measurements of the thoracic spiracular valves were made. The influence of valve action on the emission of CO_2 and water loss was recorded by flow-through measurements concurrently with the air sac pressure registration. This study provides the first documentation of how tracheal pressure cycles, resulting from periodic heartbeat reversals, contribute to respiratory gas exchange.

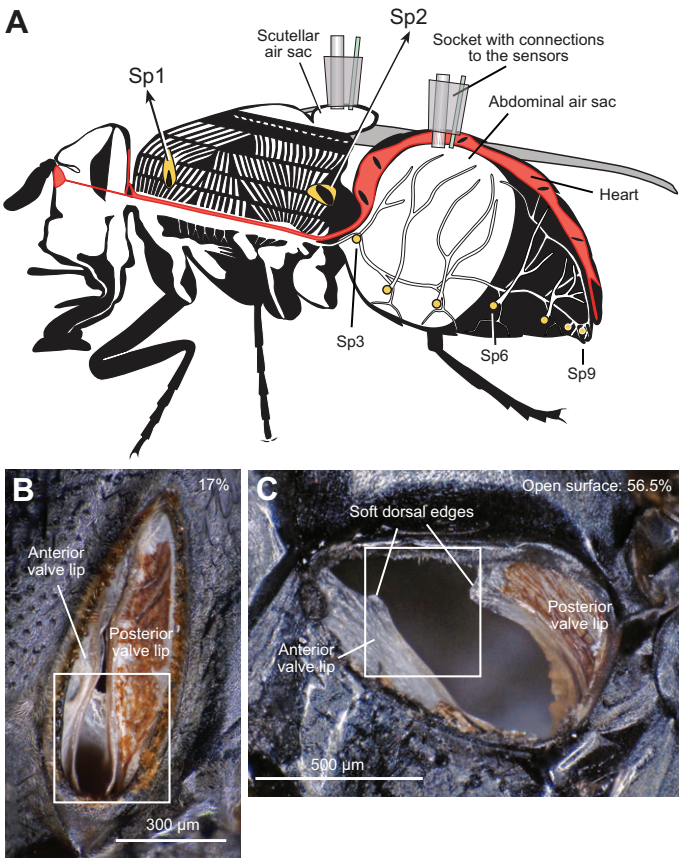
RESULTS

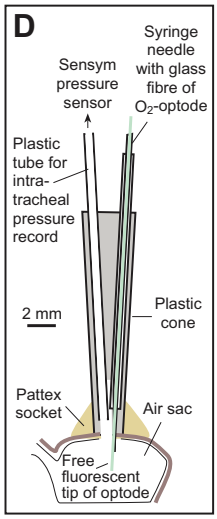
Structure of the spiracles

Callipora vicina has two pairs of thoracic spiracles, the mesothoracic Sp1 and the metathoracic Sp2. Both spiracles can be closed by valves, which consist of opposing anterior and posterior membranous lips (Fig. 1A–C). The lips are stretched and kept in tension by the underlying haemolymph. The free edges bordering the aperture are stabilized by sclerotized elastic bars, which are ventrally connected to the V-shaped muscle. In Sp2, the dorsal part of the valves is soft and flexible (Fig. 1C). The seven pairs of abdominal spiracles are very small and have a simple circular opening with loosely arranged bristles in the atrium (Fig. 1A). Their inner valves were not visible under our experimental conditions and were not analysed further. The surface of the thoracic spiracles is equipped with peritrema filter plates of ramified bristles, which protect the atrium and delicate valves behind. The filter bristles of Sp1 form a dense stable roof (Fig. 2A). Sp2 has an anterior fixed plate (Fig. 2G) and a posterior pin-jointed plate, which can be opened passively by a strong expiratory air stream (Fig. 2H). The filter plates were removed for observation of the valves. The thoracic valves are said to be closed by a muscle and to be opened by the elasticity of the sclerotized rims of the valve lips (Krancher, 1881; Hassan, 1944; Faucheux, 1973). However, our own observations and recordings suggest that these valve muscles are openers (L.T.W. and A. S. Fröhlich, unpublished).

Activity of the spiracular valves

During and immediately after CO_2 anaesthesia, the valves of Sp1 and Sp2 were widely open (Fig. 2F,N). Visual observations suggested that after recovery in quiescent blowflies, the valves were mostly closed (Fig. 2B–D,I–K). The valves opened during and especially for a while after activity such as locomotion, grooming and feeding. When ceasing activity, the constricted valves seemed to be closed. In the hours after feeding, quiescent flies opened the valves at intervals (mean 21.6±9.8 min; N=9, n=8 per fly) without visible exercise. The valves of Sp1 and Sp2 on both sides opened and closed generally synchronously, showing the same tracheal pressure in front of the spiracular openings (Fig. 3A,B). However, the degree of opening and correspondingly the pressure amplitude at the tubed spiracle could differ (Fig. 3C). The opening slit of the valves varied continuously between 0 and 8% of the maximal





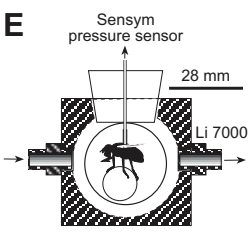


Fig. 1. Anatomy of the tracheal system in *Calliphora vicina* and the experimental setup. (A) Semi-schematic median section of *C. vicina* showing the tracheal system with the projection of the lateral spiracles (orange) and position of the sensors on the punctured dorsum above the scutellar and abdominal air sacs. (B,C) First spiracle (B), second spiracle (C) of a living fly. The filter bristles of both spiracles were removed to expose the valve lips and the aperture (the percentage of the maximum possible opening is given in each panel). The rectangles indicate the projection of the sensor area of the Si cell for recording of the opening and closing of valve lips. (D) Arrangement of the pressure sensor tube and O_2 optode connected to the scutellar air sac. (E) Flow-through chamber for measuring CO_2 and H_2O emissions and simultaneous tracheal pressure.

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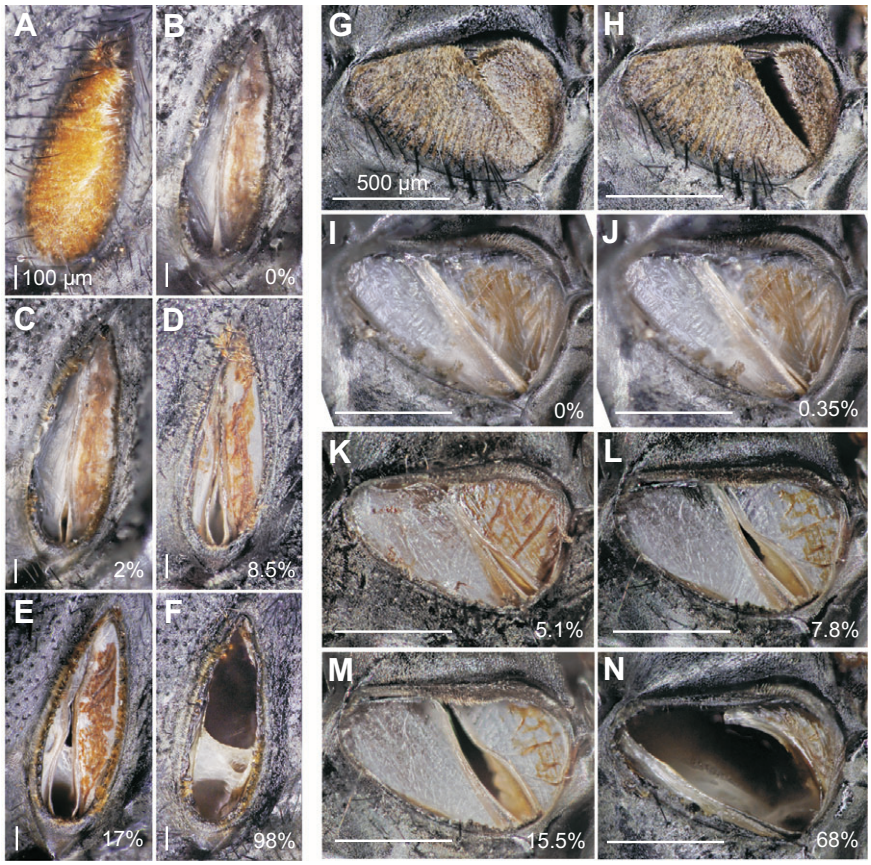

Fig. 2. Structure of the thoracic spiracles of a living *C. vicina*. (A–F) First spiracle. (A) Left Sp1 with intact peritrema. (B–F) Peritrema trichomes removed to show the spiracular valves. (B) Closed valve lips. (C) Valve lips open at the ventral base exposing 2% of the spiracle surface. (D) A drop-shaped opening increased to 8.5%, exposing the ventral tracheal stem. (E) An open surface of 17% also exposes part of the dorsal tracheal stem. (F) Both valve lips are retracted and expose 98% of both, the ventral and dorsal tracheal stems. (G–N) Second thoracic spiracle. (G) Left spiracle with closed filter plates. (H) Sp2 with abducted posterior filter plate. (I–N) Filter plates removed to show the inner valve with different degrees of opening, operated by the spiracle muscle. (I) Valve lips fully closed. (J) A 0.35% opening through a ventral gap between the anterior and posterior valve lips. (K) An opening of 5.1%. (L) An opening of 7.8%. (M) Both lips are retracted to enlarge the gap to 15.5%. (N) Valve lips are retracted and turned inwards exposing 68% of the spiracle surface.

possible open area. With widely opened valves (above 40%, Fig. 2F,N), the tracheal pressure was equilibrated with the atmosphere, and no pressure pulses could be recorded (Fig. 4). This happened especially after intensified activity.

Comparison of tracheal pressure at the spiracles and in the dorsal air sacs

The measurement of the tracheal pressure in front of the spiracles is problematic because the closing of the valves interrupts the connection of the sensor with the tracheal lumen. Moreover, the partial opening of the valves can result in local pressure differences at the individual spiracle (Fig. 3C). The sensor blocks the connection of the spiracle opening with the ambient air and may thus artificially affect the pressure of the entire thoracic system. To circumvent the possible disturbance of the spiracles, measurements of tracheal pressure were performed using intubations of the dorsal air sacs to obtain integrated values of the pressure and partial pressure of oxygen (P_{O_2}) from the thoracic or abdominal tracheal system. Basically, the pressure curves show the same pattern whether recorded at the spiracles or at the dorsal air sacs.

Passive movements of the spiracular valves

In addition to the active movements of the spiracular valves, a minute leak at the dorsal soft part of the rims of the valve lips of Sp2 could be detected (Fig. 5). This leak is difficult to see and was hitherto overlooked. During volume and pressure decrease in the thoracic haemocoel by backward heartbeat, the leak was passively widened (Fig. 5C–E). This explains why ambient air could be sucked into the thoracic air sacs and the P_{O_2} increased even though the proper valve remained constricted (Fig. 5). The minimal extension of the leak during forward pulse period could be near 0% and expand to only 1% during backward beating (N=8; Fig. 5A,B). The widening of the leak can start from ~1% and extend to 5% (N=5; Fig. 5B,C) or from ~8.5 to 13.7% (N=3; Fig. 5D,E, supplementary material Movie 1). The regular periodicity of the leakage was also measured by the changes of reflected light from the valve opening and the resulting exposure of the dark inner tracheal background (N=3; Fig. 6). In the video recordings, it was seen that the valve lips vibrate in the frequency of the backward pulses, while they continuously increased the leak. The haemolymph on the rear of the valve lips is sucked in by the conical heart chamber connected by the lateral venous channels with the meta-thoracic spiracular region, as in *Drosophila* (Wasserthal, 2007) (L.T.W., unpublished data).

Oxygen uptake is concurrent with heartbeat reversals

Oxygen fluctuations in the scutellar air sacs were measured in intubated flies with simultaneous registration of intra-tracheal pressure or heartbeat. As postulated, the compensatory pressure decline in the air sacs of the anterior body by the negative pulses during backward beating was correlated with an oxygen increase in the anterior body (Figs 7, 8). At an ambient temperature (T_a) of 21°C, the P_{O_2} ranged between a lower mean of 17.5±1.1 kPa and an upper mean of 18.9±1.1 kPa. (N=17, evaluated sequences: 1907; time: 20 h). At low metabolism in hibernating flies at a T_a between 3 and 19°C, intratracheal pressure cycles continued with a coincident O_2 increase during backward pulse periods leading to a mean P_{O_2} of between 16.9±4.05 and 17.9±3.2 kPa (N=7, n=523; Table 1, supplementary material Table S1). The single lowest P_{O_2} of 4.5 kPa was measured at an intermediate T_a of 10°C. No general reduction of P_{O_2} in hibernating flies at low ambient temperature was recorded (supplementary material Table S1). The O_2 rise (ΔP_{O_2} per

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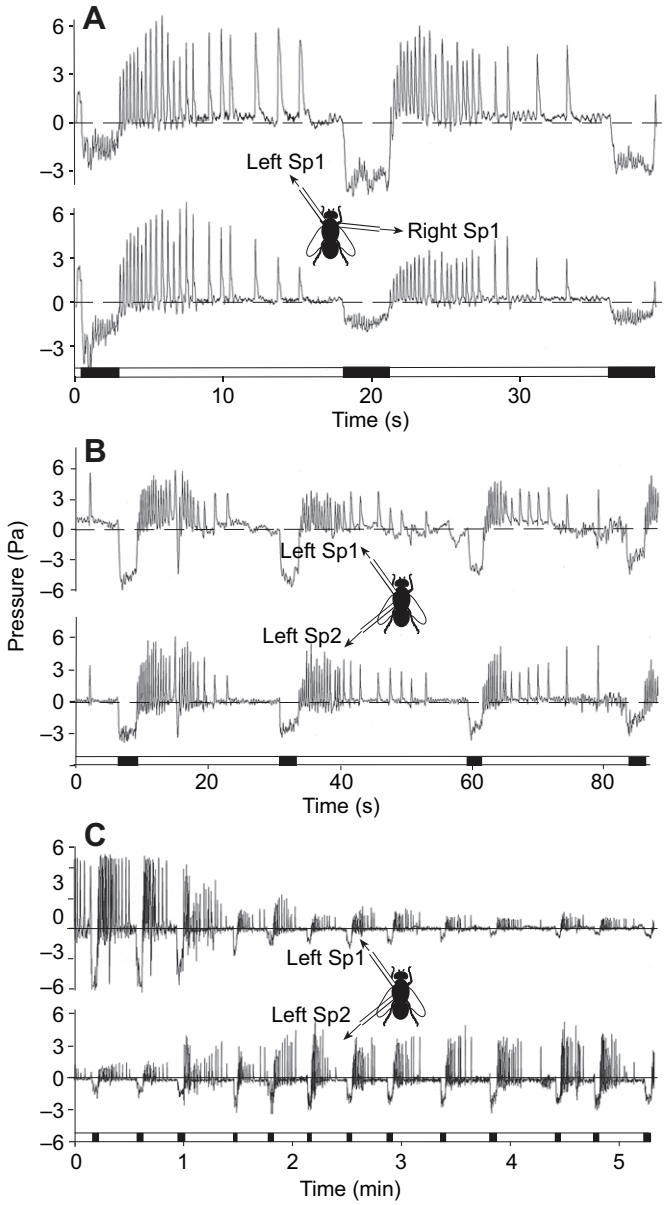

Fig. 3. Relative atrial pressure at ipsilateral or contralateral spiracles in resting male *C. vicina* [Male (M) 15/2000 (15/00) at 22°C]. The periodic pulse sequences reflect the heartbeat reversals. Black bars: negative pressure pulse periods corresponding to backward heartbeat periods. (A) The similar amplitude of the pressure pulses at the left and right anterior spiracle indicates an almost identical opening of the valves at both sides. (B,C) Relative atrial pressure at the left anterior and left posterior thoracic spiracles. (B) The similar amplitude of the pressure pulses indicates an almost identical opening of the valves in both segments as in A. (C) The amplitude of the pressure pulses showing an inversely proportional change at the anterior and posterior thoracic spiracles when the valves behave differently. 0=atmospheric pressure.

peak) during each negative pulse period ranged between 0.1 and 2.5 kPa with a mean of 0.5±0.2 kPa at 21°C. During sustained quiescence with Sp2 leaking, the P_{O_2} remained constant at a high level (18.57±1.09, *N*=19), and no decrease could be seen towards the end of a several minutes-long period of resting heartbeat cycles. The mean P_{O_2} was also high when the O_2 peaks were weak and disappeared in the noise (mean 18.4±1.3 kPa, *N*=14, 20–30 sequences per fly). In a few cases, when the backward pulses were

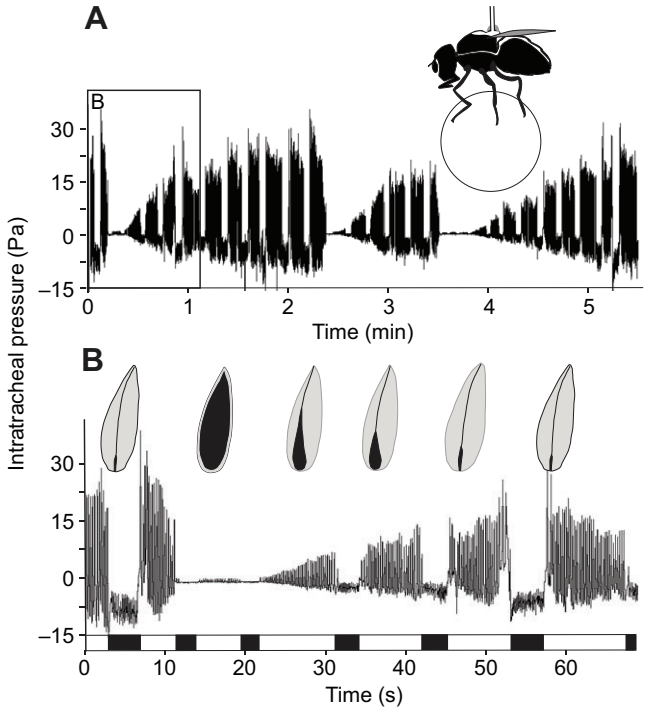

Fig. 4. Intratracheal pressure changes in the scutellar air sac attributed to spiracle action in *C. vicina* [Female (F) 13/2009 (13/09) at 22°C]. (A) A series of 31 heartbeat sequences interrupted by three phases of zero (=ambient) pressure: black bars, negative pressure pulse periods indicating backward heartbeat. Tracheal pressure equalizes with atmospheric pressure when the spiracles are fully open. Pulses cannot be resolved. Inset shows fly on a Styrofoam ball used for feeding and calming of the fly. (B) Detail of A. Re-establishment of pressure pulses after 20 s with decreasing opening of spiracle 1 (inset diagrams of spiracles taken from frames of video recordings).

exceptionally omitted and pressure pulses remained positive for 4.8±0.28 min (*N*=3; Fig. 8B), the mean P_{O_2} decreased by about 3±0.5 kPa in the thorax. After the reappearance of the rhythm with backward pulse periods and closed but leaking spiracles, the P_{O_2} returned to the original, higher values (Fig. 8B at 18:20 h).

Measurements in the abdominal air sacs showed the reciprocal correlation of O_2 increase during forward pulse periods with a rise in ΔP_{O_2} of 0.96±0.45 Pa (*n*=46 sequences), which produced a mean P_{O_2} level of 18.4±0.7 (*N*=3 females; evaluated sequences: 328 and time: 3.3 h; Fig. 9).

Convective CO_2 micro-bursts concurrent with heartbeat reversals

The CO_2 output of the entire insect was measured by flow-through respirometry combined with recording of the intra-tracheal pressure of the scutellar air sac (*N*=14, evaluated sequences: 2287 and time: 13 h). The CO_2 micro-bursts ranged between a minimum of 4.4 nmol s^{-1} g^{-1} and a maximum of 28.8 nmol s^{-1} g^{-1} (Table 2, supplementary material Table S2). The pressure increase in the scutellar air sacs corresponding to periodic haemolymph accumulation in the thorax was in most cases correlated with a CO_2 micro-burst (Fig. 10A). The mean burst amplitude was 6.4±1.5 nmol s^{-1} g^{-1}. Although the O_2 rise in the anterior body occurred during backward pulse periods and in the abdomen during forward pulse periods, the moment of maximal CO_2 emission could change for some time within measurements in the same fly, possibly depending on the phases of digestion of crop contents (Fig. 10B).

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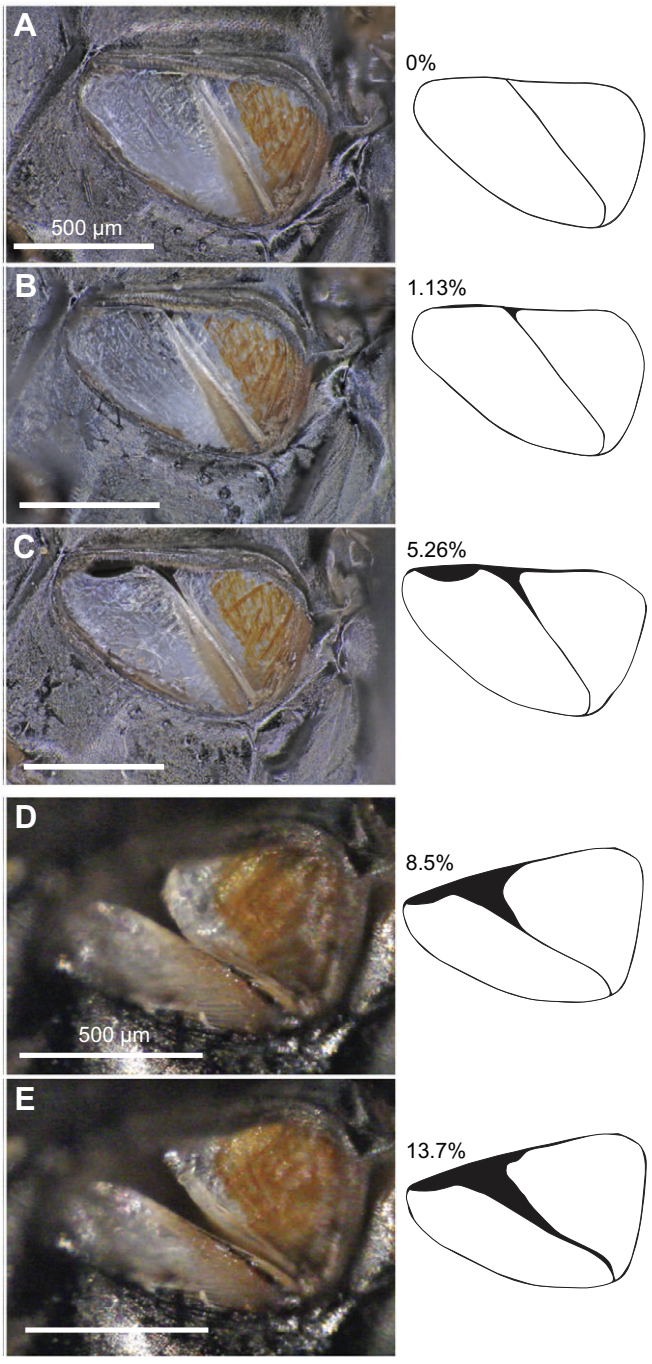

Fig. 5. Leakage at the second spiracle varies with the periodic change of haemolymph volume in the thorax. Filter plates were removed. Left column: photographs of living flies (A–C) and video frames (D,E). Right column, schematic representation of analysed open areas. (A) Valve lips fully closed. (B) Leak very small (1.13%). (C) Leak at the dorsal soft edges of the lips increased to 5.26%. (D) Leak of 8.5% at the end of a forward pulse period, which at (E) increased at the end of a backward pulses period to 13.7%. See supplementary material Movie 1.

The coincidence of the CO_2 maxima with one of the pulse directions was not as reliable as the O_2 increase in the thorax and abdomen. As the CO_2 recordings by flow-through respirometry comprise the CO_2 emission of the entire body, it can only be deduced from the pressure conditions whether the emission came from the anterior or from the posterior body or from both parts. In three of 14 flies, distinct CO_2 bursts occurred during forward pulse periods and backward pulse periods (Fig. 10C, supplementary material Table S2). In these cases, the one burst during forward pulse periods was attributed to expiration of the anterior body, and the other one during backward pulse periods to expiration of the abdomen. In some sequences, both bursts were equally strong and fused. Although being generally cyclical, the CO_2 emission never decreased to 0.

CO_2 macro-bursts and oxygen drop during full spiracle opening

In addition to the cardiogenic CO_2 micro-bursts in the leaky phase, during active opening of all thoracic spiracles, the residual CO_2 was released as a macro-burst with a high mean amplitude of 273.5±151.4 nmol s^{-1} g^{-1} (N=9, 7–8 per fly at 22°C; Fig. 10D). The inter-burst phase between the CO_2 macro-bursts lasted 21.55±9.82 min. The amplitude of the macro-bursts was up to 683.8 nmol s^{-1} g^{-1} in well-fed quiescent individuals. The resting periods were often interrupted by phases of running on the Styrofoam ball, grooming or regurgitating and re-imbibing the crop contents, accompanied by irregular CO_2 release omitting the cyclical macro-bursts. All flies exhibited long phases of intermittent activity without the cyclical CO_2 macro-bursts. As an unexpected result, the P_{O_2} in the scutellar air sac dropped by ΔP_{O_2} of 2.75±1.16 kPa (N=11) when the spiracles fully opened (Figs 11, 12). This is the contrary to what one would have expected (see Discussion).

DISCUSSION

Heartbeat reversals cause oxygen inflow by negative pressure and leaking spiracles

The simultaneous intratracheal P_{O_2} measurements and pressure measurements confirm the hypothesis that the cardiogenic negative pressure periods cause active inspiration. Because the negative intra-tracheal pressure arises in the anterior body during backward (retrograde) periods and in the abdomen during forward (anterograde) pulse periods (Wasserthal, 2012), it is evident that the heartbeat reversal is the causal force of this mechanism. The periodic P_{O_2} increase cannot be explained by a mere physical effect due to pressure changes. Under negative pressure in a closed system, the P_{O_2} should decrease and not increase. Moreover, the P_{O_2} reduction during pressure decrease is so small that it does not significantly counteract the observed P_{O_2} rise. The determined pressure-dependent physical P_{O_2} changes between 0.02 and 2 Pa have an effect of only 0.004 and 0.4%, respectively, with regard to the higher mean ΔP_{O_2} rise of 0.5±0.2 kPa.

It is a remarkable result that the P_{O_2} peaks occur and the relatively high mean P_{O_2} (17.5–18.9 kPa) remains constant, although the thoracic spiracles are constricted in the neuromuscular sense. However, a critical inspection of the spiracular valves revealed a very small leak of Sp2 widening in the course of the backward pulse periods of the heart. It is this leakage, increased by the negative pressure, that allows the respiratory inflow and rise of the tracheal P_{O_2}. At the first spiracle, no comparable leak could be detected. The possibility cannot, however, be fully excluded that the ventral slit of the Sp1 valve is slightly opened below 1%. The periodic P_{O_2} rise during the negative pressure periods (forward pulses) in the abdomen is assumed to function in a similar way to spiracle leakage.

Comparison of the cardiogenic gas exchange with the passive suction ventilation in other insects

Passive suction ventilation (PSV) is the result of the discontinuous gas exchange cycle (DGC) based on the constricted phase, flutter

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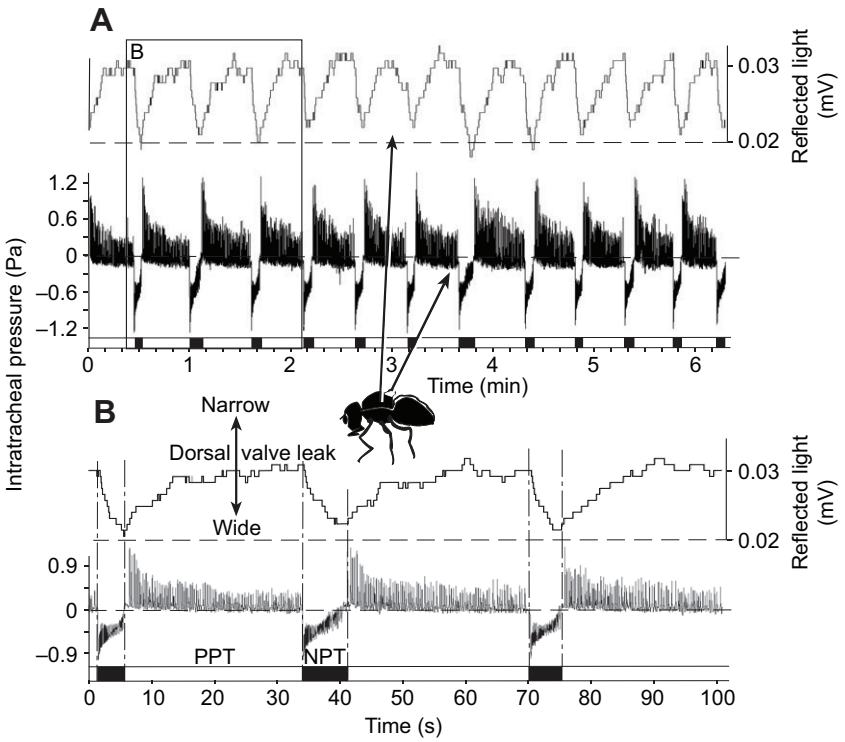

Fig. 6. Influence of the heartbeat reversals on the dorsal valve leakage of spiracle 2 in *C. vicina* (F7/11 at 22°C). (A) Upper trace: reflection of valve lips on Si photocell. During widening of the leak the reflective surface of the lips is reduced and the dark background behind the leak increases. Lower trace: relative tracheal pressure pulses. Negative pressure pulses are caused by backward heartbeat (black bars) and positive pressure pulses are caused by forward heartbeat (white bars). (B) Detail of A. PPT, positive pressure pulse periods in the thorax; NPT, negative pressure pulse periods in the thorax.

phase and open phase (CFO) of the spiracles described in lepidopteran pupae (Schneiderman, 1960; Levy and Schneiderman, 1958; Brockway and Schneiderman, 1967). During the C-phase, the P_{O_2} decreases to a low value of 3–7 kPa because of O_2 consumption, and the tracheal pressure becomes sub-atmospheric. When in the following F-phase the spiracles open briefly, the tracheal pressure increases to nearly atmospheric values and fresh air is sucked in, preventing the P_{O_2} from sinking below the above fairly constant low value. This gas exchange by simultaneous diffusion and inward convection of O_2 is based on a great difference in partial pressure and hydrostatic pressure (Miller, 1974). It retains CO_2 and H_2O while allowing maximal O_2 uptake in a N_2 equilibrium of outward diffusion and inward convection (Kestler, 1985).

In the cardiogenic gas exchange of *C. vicina*, air is also sucked in by the negative intratracheal pressure, but it is not supported by a high diffusive gradient of P_{O_2} between the tracheal lumen and the atmosphere as in the PSV of the pupae. The suction mechanism in *C. vicina* is based on the negative pressure in the haemocoel, which expands the tracheal lumen. The inspiration by tracheal volume increase enables the airflow to reach the terminal tracheoles of the blindly ending tracheae or air sacs. By contrast, in the classical PSV in lepidopteran pupae, the suction force arises by the higher molar oxygen uptake than molar CO_2 release due to buffering of metabolic CO_2 in the tissues and haemolymph and delayed transition into the tracheae in the O-phase (Levy and Schneiderman, 1958). The resulting negative intratracheal pressure in the C-phase is partly responded to by the reduction of the tracheal volume, putting the compliant tracheae under tension and shortening the abdomen. The opening of the spiracles in the F- and O-phases leads to a relaxation of the tracheae and abdomen resulting in volume and length increases, respectively, with the consequence of inspiration. Both suction mechanisms avoid stagnant air and the dependence on diffusion alone.

The cardiogenic form of active suction ventilation is performed by negative pressure periods alternating in the anterior body and abdomen and passively adapting spiracle leakage. This stereotypical rhythm fulfils a similar job as the flutter phases of the CFO-type in other insects, by enabling a longer lasting convective and diffusive O_2 uptake, whereas CO_2 and H_2O have to diffuse against the inflowing air. In the flutter phases, an active role of the spiracles in regulation of the inspiratory airflow is assumed, and the term ‘flutter’ describes the sudden neuromuscular movements of the spiracular valves (Miller, 1981). By contrast, the proper valve mechanism in the leaky phase in *C. vicina* is not fluttering as the valve remains constricted, and the Sp2 leak widens gradually and vibrates with heart pulses in the course of each backward pulse period of the heart and narrows in the course of the forward pulse period. A similar spiracular behaviour can be deduced in the abdomen from the O_2 peak during the negative pressure period in the abdominal air sac. A fluctuating leakage of the abdominal spiracles could not be detected here because of their microscopic dimension.

Cardiogenic CO_2 micro-bursts and CO_2 macro-bursts

A difference between the CFO-type and the cardiogenic gas exchange is the convective release of CO_2 in that compartment, which, because of the positive pressure periods, receives an increasing haemolymph volume, which is compensated for by the decreasing tracheal volume, and leads to a local and temporal separation of the interburst O_2 uptake and CO_2 micro-burst in the anterior body (Fig. 10A) and in the abdomen (Fig. 10B) or alternating in both (Fig. 10C). Independent of the cardiogenic gas exchange in *C. vicina*, macro-bursts of CO_2 emissions occur in longer intervals. They are reminiscent of the cyclical gas exchange (flutter–burst-type) in other insects with a long inter-burst phase and a short macro-burst phase. The inter-burst phase corresponds to the leaky phase in *C. vicina* with a variable number of heartbeat sequences. A correlation between inter-burst duration and number of heartbeat sequences, corresponding to the cardiogenic micro-bursts, could not be found. With a sequence duration of 27.5–39 s (data from Tables 1, 2) between 32 and 46 heartbeat sequences could occur during one inter-burst phase with a mean duration of 21 min.

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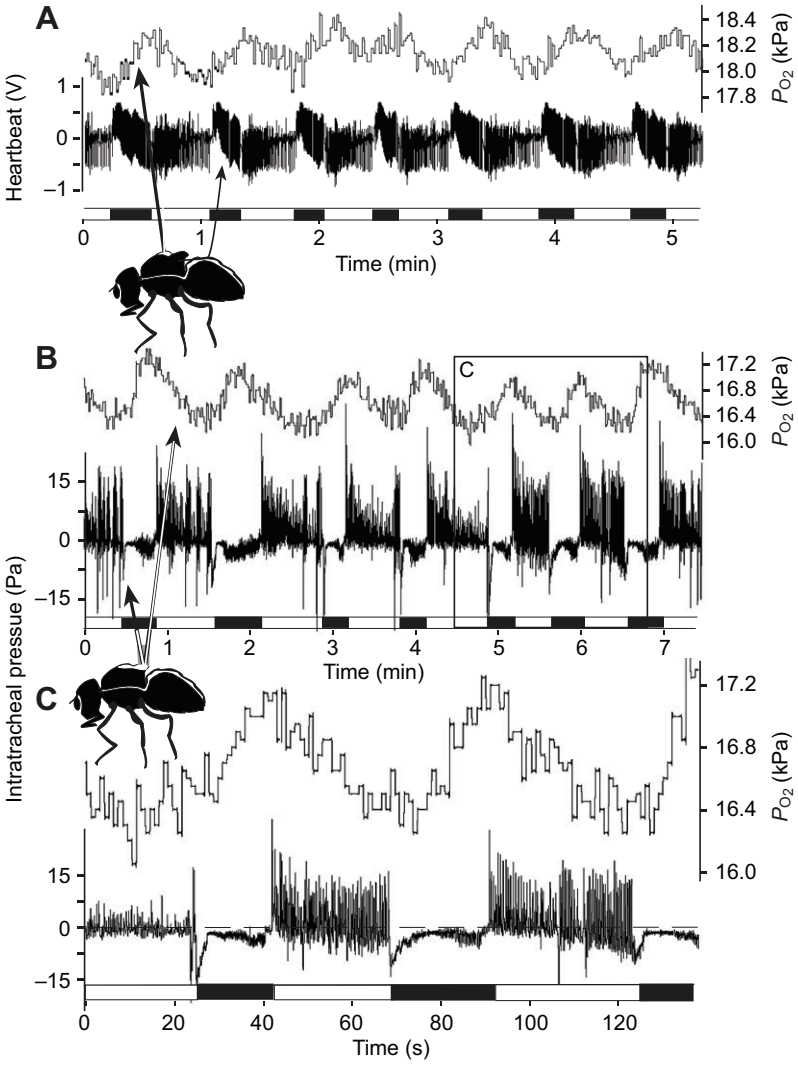

Fig. 7. Oxygen uptake is concurrent with heartbeats. (A) P_{O_2} in the scutellar air sac (upper traces) and simultaneous myographic recording of heartbeat (lower trace) in male *C. vicina* (M15/09 at 22°C). During backward pulse periods (black bars), the P_{O_2} increases. (B,C) Simultaneous recording of intra-tracheal P_{O_2} and pressure in the scutellar air sac (F6/08 at 22°C). (B) Survey of seven sequences. (C) Detail of B. The P_{O_2} increases during the negative pressure periods (backward pulse periods; black bars).

P_{O_2} drop by accumulation of intratracheal CO_2 during spiracle opening

The P_{O_2} drop in the scutellar air sac during the phases of full spiracle opening differs from published results in other insects with cyclical constriction, flutter and burst (CFB) phases of the spiracles, such as in lepidopteran pupae, beetles or cockroaches: the tracheal O_2 rises when the CO_2 is released as a macro-burst in the open phase (Punt et al., 1957; Levy and Schneiderman, 1966; Lighton, 1988; Hetz et al., 1994; Matthews and White, 2011). O_2 uptake occurs simultaneously with CO_2 release and additional small amounts during the flutter phase. In *C. vicina* the O_2 uptake seems to be fully disconnected from the CO_2 macro-burst.

The probable reason for the P_{O_2} drop during opening of the spiracles is the accumulation of CO_2 in the tracheae during the transition from the dissolved phase in the tissues and haemolymph into the gaseous phase just before and during release through the spiracles. Intra-tracheal CO_2 measurements inside the flies are not available, but it is known that the haemolymph pH becomes less acidic during the open phase in butterfly pupae (Hetz and Wasserthal, 1993) and in cockroaches (Matthews and White, 2011). The alkalosis in the haemolymph is an indication that the relative share of CO_2 increases the tracheal P_{CO_2} and the total tracheal pressure, and leads to an enhanced outflow of O_2 into ambient air, which lowers the concentration of the O_2. Measurements of O_2 consumption in lepidopteran pupae showed a similar O_2 drop ascribed to CO_2 bursts (Jõgar et al., 2011). The clear separation of the CO_2 burst during the open phase and the O_2 uptake during the leaky phase suggests that the active spiracle opening serves for CO_2 release. This separation confirms results in *Hyalophora* pupae (Levy and Schneiderman, 1958; Buck, 1962) and in the grasshopper *Taeniopoda eques*. In both insects the spiracle opening is triggered by a certain threshold of P_{CO_2} (Levy and Schneiderman, 1966; Harrison et al., 1995).

Leaking gas exchange for water vapour retention

The persistent, relatively high P_{O_2} of 17.5–18.9 kPa in the phases with constricted, but leaking spiracles in quiescent *C. vicina* is contrary to the hypothesis that the constriction phase prevents toxic damage by O_2 radicals under low oxygen demand, which has been argued in connection with lepidopteran pupae (Hetz and Bradley, 2005). In adult flies, the maintenance of a high P_{O_2} with leaking spiracles favours the classical hypothesis of water retention, which has been suggested for saturniid moth pupae (Buck, 1962; Miller, 1974), and for Blattodea (Kestler, 1985; Schimpf et al., 2009), in which, under normoxic conditions, the P_{O_2} lies above 15 kPa (Matthews and White, 2011). As in any active suction ventilation during inspiration over a short valve distance, water vapour cannot diffuse outwards against the inflowing air stream (Kestler, 1985). But in *C. vicina*, water can be lost during positive pressure periods, which cause several CO_2 micro-bursts during the interburst (Fig. 10A–C). The difference between the cardiogenic micro-bursts

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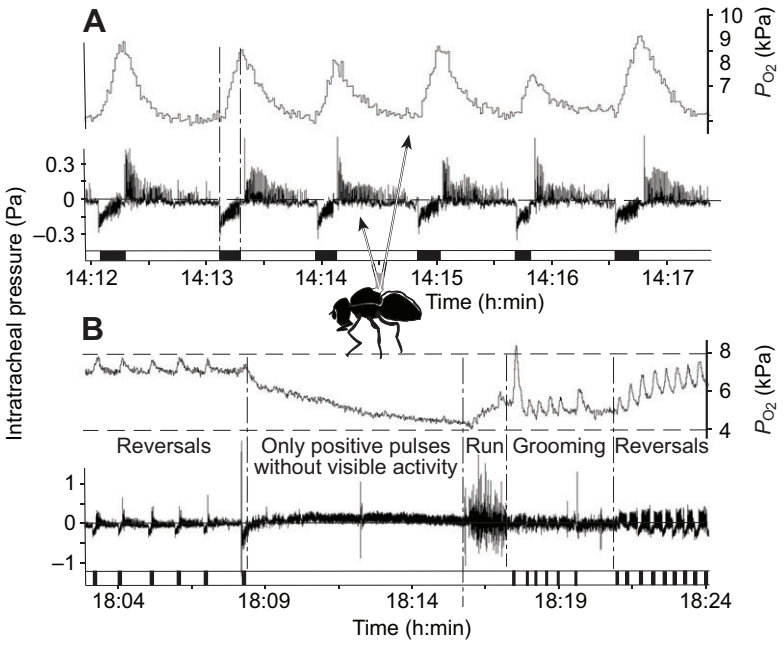

Fig. 8. Cyclical P_{O_2} and pressure changes in the scutellar air sac during rest and different activities. The increase in P_{O_2} during negative pressure pulse periods (black bars), due to the backward beating heart in female *C. vicina* (F33/09 at 14°C). (A) Regular periodicity of pressure and P_{O_2} changes. (B) Periodicity with different cycle lengths before and after a longer phase of positive pressure pulses and phases of running and grooming. In the long phase without negative pulse periods, the P_{O_2} decreases without any visible activity. It increases during the following running and grooming activities and returns to a higher level after re-established heartbeat reversal. Black bars, backward pulse periods.

and the macro-bursts is a convective gas exchange versus a diffusive gas exchange of the macro-burst. As water molecules diffuse quicker than CO_2 molecules, a diffusive gas exchange of CO_2 should lead to 59.6% higher water loss than a convective gas exchange of the same amount (Kestler, 1983; Kestler, 1985). This water retention can be deduced from the data in Fig. 12. A first comparison of the ratio of H_2O evaporation per 1 nmol of CO_2 emission during the interburst of 12 cardiogenic micro-bursts, which amounts to 0.33 nmol g^{-1} with that of one macro-burst, which amounts to 0.79 nmol g^{-1} H_2O per 1 nmol g^{-1} of CO_2, results in a 58.2% higher diffusive water loss in the macro-burst. This preliminary evaluation confirms the above hypothesis that the cardiogenic mechanism is advantageous to withhold water as the number and duration of diffusive macro-bursts is reduced in favour of prolonged interbursts with convective CO_2 micro-bursts.

Cardiogenic ventilatory mechanism in other insects

There are few known examples of cyclic gas exchange in other flies, and no comparison exists with the periodic heartbeat reversals. In *Drosophila melanogaster*, CO_2 bursts occur in populations selected for desiccation resistance with a burst cycle frequency of 1.2 per minute (Williams et al., 1997). The CO_2 release during the 'closed' phase was discussed with regard to a possible leakage of the spiracles. In mosquitoes a similar burst cycle frequency of 1.1 per minute was recorded at a flow rate of 1000 ml min^{-1} at 20°C (Gray and Bradley, 2006). In tsetse flies, CO_2 macro-bursts have been recorded with a cycle frequency of 0.054–0.080 Hz, which is equal to 3.2–4.8 cycles min^{-1} (Terblanche and Chown, 2010). All these burst frequencies are high when compared with *C. vicina*. Fewer heartbeat sequences or only a single one might occur during one of these relatively short inter-burst phases. *Drosophila* with a heartbeat sequence length of 19–25 s (Dulcis and Levine, 2005; Wasserthal, 2007) and mosquitoes with a sequence length of ~20 s (Glenn et al., 2010) would perform two to three cardiogenic cycles per one inter-burst. In contrast to these fast frequent macro-burst cycles, *Drosophila mimica* releases CO_2 bursts with pronounced flutter phases of the classical CFO cycle alternating with long-lasting leaky phases of ~30 min (Lehmann and Schützner, 2010). It is probable that these leaky phases are accompanied by numerous heartbeat reversals and reflect the cardiogenic ventilatory mechanism.

Periodic heartbeat reversal is a widespread phenomenon in insects (Gerould, 1929; Jones, 1977; Wasserthal, 1996), and it is predicted that it contributes to gas exchange in all cases in which the haemolymph is periodically shifted between the anterior body and abdomen. In contrast to the flies described here, in adult giant silk moths, the CO_2 emission cycle is strongly coupled with the heartbeat sequence (Wasserthal, 1996) with periodic expansion and contraction of abdominal movements and superimposed bouts of peristaltic contractions at the end of each backward pulse period, which are coordinated with metachronic closing of the abdominal spiracles (Wasserthal, 1981).

Conclusions and perspectives

Tracheal ventilation in quiescent *Calliphora* turned out to be mostly a side effect of periodic heartbeat reversal causing the changes in tracheal volume and appropriate spiracular valve leaks. Inspirations are shown to be only cardiogenic, due to backward beating through increasingly leaking valves in the thorax and during forward

Table 1. O_2 concentration and peak amplitude per cardiac sequence measured in the mesoscutellar air sac at different ambient temperatures

		Heartbeat sequence					
	N	No. analysed	Time (h)	Frequency (cycles min^{-1})	Duration (s)	P_{O_2} range (kPa)	ΔP_{O_2}/peak amplitude (kPa)
Mean T_a 10°C	7	523	17	0.73±0.5	82±56	16.9±4.0–17.9±3.2	0.5±0.1
Mean T_a 21°C	17	1907	20	1.53±0.61	39±15.6	17.5±1.1–18.9±1.1	0.5±0.2

P_{O_2}, partial pressure of oxygen; T_a, ambient temperature.
Values are means ± s.d.; *N*, number of flies.

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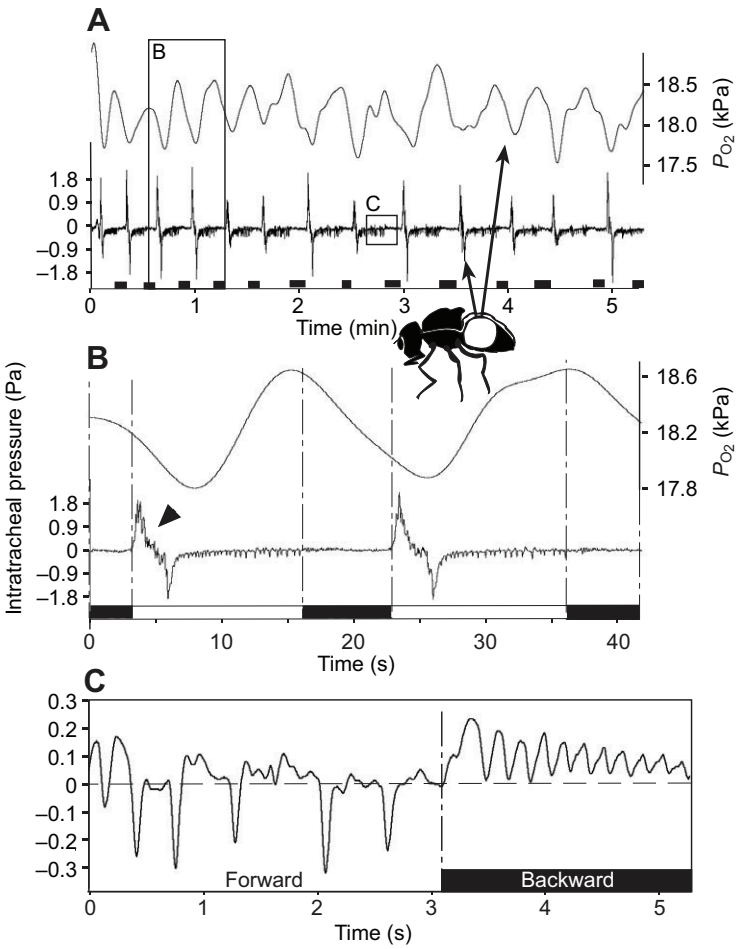

Fig. 9. Abdominal air sac pressure and P_{O_2} in *C. vicina* (F31/09 at 23°C). (A) A survey of 13 sequences. (B) Detail of A. The P_{O_2} increases during forward beating and decreases during backward beating (black bar) and during the pumping stroke (arrowhead) at the beginning of the forward beating period. (C) Detail of A. Transition from forward pulses with negative peaks to backward pulses with positive peaks. The P_{O_2} and air sac pressure are inversely proportional to conditions in the thorax.

heartbeat in the abdomen. The expirations follow mostly during forward heart pulses. Single abdominal pumping strokes coincide with the onset of each forward pulse period of the heart during the interburst (Wasserthal, 2012). They have no measurable effect upon the gas exchange, whereas in other insects abdominal ventilatory movements are typically volleys during the macro-bursts. In *C. vicina* the residual CO_2, which is retained in the tissues and haemolymph during the long interburst, is released as a macro-burst during open phases. Thus, at rest, the respiratory gas exchange in the flies is water saving and energetically economic, because no special ventilation movements and spiracle muscle activity, as in the flutter and open phase of other insects, are required. This provides reserves for the high metabolic demands during flight, which in *Drosophila* is matched by continuous active opening of the spiracles (Lehmann, 2001). Whether in *C. vicina* during flight, periodic haemolymph shifts by heartbeat reversals continue and whether they contribute to the gas exchange will be addressed in a future study.

In Lepidoptera and scarabaeid beetles, the elastic tracheae enable a tidal haemolymph flow in the wings and elytra, respectively, functioning as counterforce to the periodic heartbeat reversals and intermittent and coordinated action of the accessory pulsatile organs (Wasserthal, 1982; Wasserthal, 1996). In flies the role of the accessory pulsatile organs in the cyclic haemolymph supply and the functional and structural adaptations of the tracheal system in the gas exchange still need to be analysed.

MATERIALS AND METHODS

Animals

Blowflies (*Calliphora vicina* Robineau-Desvoidy 1830) from the field and their offspring were used for experiments. They were treated and fed as

Table 2. Cyclical CO_2 emission of entire flies (mean mass 84.5±16.4 mg) recorded by flow-through respirometry

Type of CO_2 emission	T_a (°C)	*N*	Heartbeat sequence/interburst cycle: No. analysed	Time (h)	Frequency (cycles min^{-1})	Duration	CO_2 emission range (nmol s^{-1} g^{-1})	CO_2 mean burst amplitude (nmol s^{-1} g^{-1})	No. CO_2 bursts during: PPT	NPT
Cardiogenic micro-burst Sp2 leaking	23	14	2287	13	2.18±0.78	Sequence 27.5±9.8 s	4.4–28.8	6.4±1.5	14	3
Macro-burst Sp1+Sp2 open	22	9	69	24	0.36±0.16	Interburst 21.5±9.82 min	34.2–683.8	273.5±151.4	Not correlated with certain pulse periods	

Values are means ± s.d.
N, number of flies; NPT, negative pressure pulse periods in the thorax; PPT, positive pressure pulse periods in the thorax; T_a, ambient temperature.

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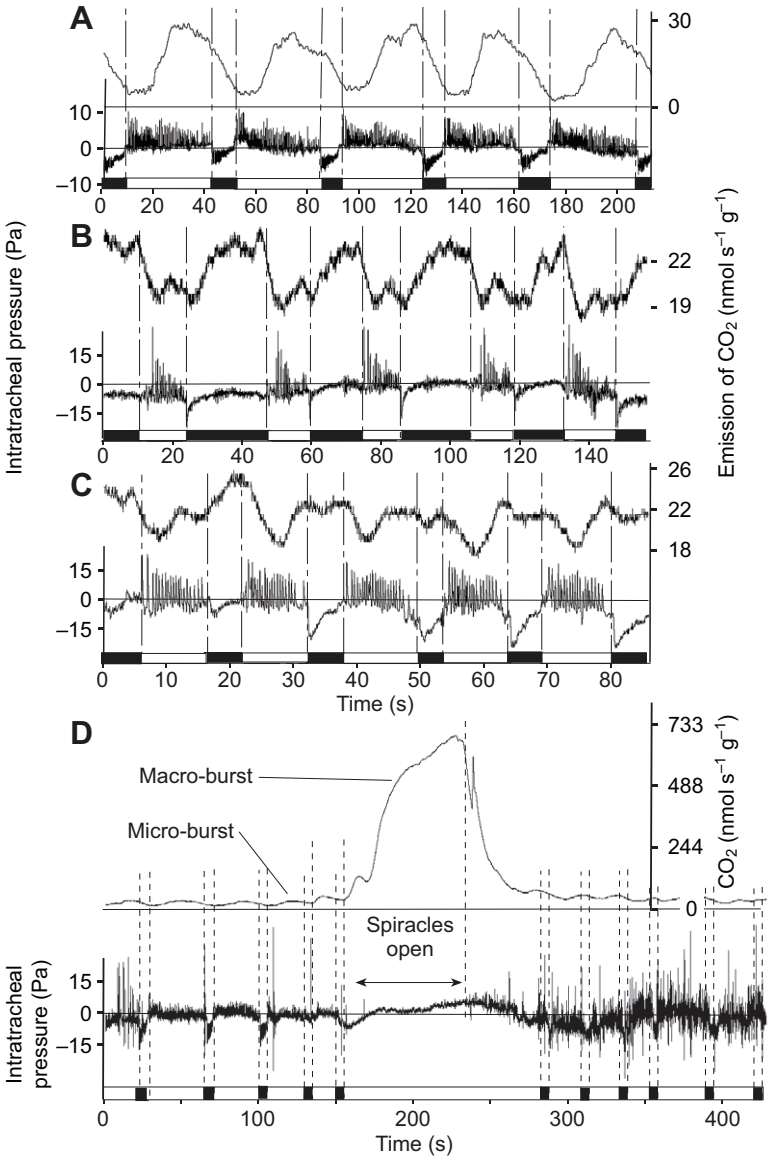

Fig. 10. Flow-through respirometry measuring CO_2 emission concurrent with intra-tracheal pressure of the scutellar air sac. (A–C) During the leaky phase; (D) during open phase. (A) Typical cyclical CO_2 micro-bursts correlated mostly with the forward pulse period (white bars). During the backward pulse period (black bars), the CO_2 emission decreases (female F9/09, 54 mg). (B) In a gravid well-fed female, the main CO_2 peak partly coincides with the backward period, which shows a less negative pressure, and a smaller CO_2 peak occurs during forward pulse periods. (C) Some hours later, the CO_2 emission peaks became equal during forward and backward pulse periods (F17/09 in B and C, 110 mg at 22°C). (D) CO_2 macro-burst during spiracle opening and slight pressure increase above ambient without pressure pulses in the scutellar air sac (F9/09 at 22°C).

described in a previously published paper (Wasserthal, 2012). The flies were anesthetized with CO_2 gas only for fixation and surgical treatment, but not during measurements. The mass of a well-fed adult was in the range 53–120 mg (mean ± s.d., 84.5±16.4 mg). It diminished by 8–20 mg when fasting for 24 h at a temperature of 20–23°C and relative humidity of 60–82%.

Recording the intratracheal pressure and heartbeat

In order to see whether the thoracic spiracles open or close simultaneously, the pressure at two spiracles (Sp1 and Sp2) in 12 flies was measured, either on the same side or on opposite sides of the same segment. Plastic tubes were glued with Pattex (Henkel, Düsseldorf, Germany) and sealed in front of the peritrema with Fixogum rubber cement (Marabu, Tamm, Germany), leaving the atrium and valves intact. Thereby these prae-spiracular measurements recorded the atrial pressure conditions, which were expected to be identical with the intratracheal pressure when the valves were open.

For measurement of the tracheal pressure inside the body, the dorsal cuticle with the underlying air sacs was perforated and connected with a bi-tubated plastic cone using Pattex, which allowed the fly to be handled and clamped in the apparatus while being simultaneously connected to the pressure sensor (Sensym SCXL 004 DN, Sensortechniques, Puchheim, Germany) and to the O_2 optode (Fig. 1). The positive or negative pressure pulses at the spiracle and in the scutellar and the abdominal air sacs reflect the activity and direction of the heart pulses and were used as the reference for the periodic heartbeat reversal and the resulting haemolymph shift between thorax and abdomen. This method of pressure measurements has been described in detail and compared with electrophysiological records in a previously published paper (Wasserthal, 2012). The dead space of the sensor of 25 µl and the 48 mm long tube connection to the spiracles or air sacs resulted in a 50% attenuation of the pressure signal. This was considered in the scaling of the curves. The response time was 7–10 ms and the time constant was 30 ms. In some flies the heartbeat was measured by extracellular electrical resistance myographs, as described previously (Wasserthal, 2012).

Recording of intratracheal oxygen

In addition to the dorsal pressure sensors, the flies were equipped with a fibre-optic optode (Microx TX3 AOT, PreSens, 93053 Regensburg, Germany). The tapered tip (diameter 50 µm) of this fibre was oriented directly above the perforation or inside the scutellar or abdominal air sacs, arranged beside the air pressure tube in the bi-tubated adapter cone (Fig. 1D). The measurements were run under controlled temperature, between 20 and 23°C, and with hibernating flies in an outside Faraday cage at ambient temperature between 2 and 19°C (Table 1, supplementary material Table S1). The sampling rate of the optode was 1 Hz. Response time was 40 ms and the time constant (interval from 17.4 to 20 kPa) in the experimental setup was

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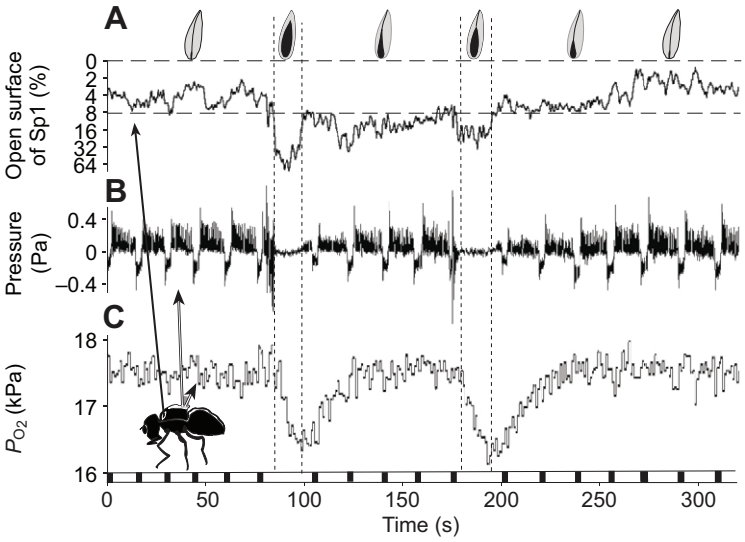

Fig. 11. Influence of neuromuscular opening of spiracle 1 on intratracheal pressure and P_{O_2} in the scutellar air sac (F7/11 at 22°C). (A) Observed opening of Sp1 and the reflected light from the valve lips recorded by a Si photodiode. When the valve opens, less light is reflected by the exposed dark tracheal hole. (B) Periodic intra-scutellar pressure pulses, which are positive during forward heartbeat and negative during backward heartbeat. When the Sp1 fully opens, the pressure approaches atmospheric pressure (0 kPa) and no periodic pulse periods are visible. (C) P_{O_2} drops during spiracle opening and is restored when Sp1 closes.

1.5 s. Calibrations in the O_2-free and ambient atmosphere were repeated before and after each experiment. The stability of the optodes allowed continuous use over several weeks without significant reduction in sensitivity and only slow, gradual loss in response time.

The influence of pressure changes on the P_{O_2} was checked by simulation experiments. In the first series, a microlitre syringe instead of the fly was combined with the pressure sensor and the optode. Doubling the syringe volume (45 µl + dead space volume of 10 µl) by 50 µl at T_a 22°C in the closed system led to a P_{O_2} decrease from 19.8 to 9 kPa, a value that would also be expected theoretically. This shows that the measured increase in P_{O_2} in the fly experiments during pressure decrease can only be explained by an inflow of ambient air into the open tracheal system.

In a second simulation series, the influence of pressure changes on the O_2 optode signal was tested in a closed 20 ml chamber. In the pressure range of 0.01–10 Pa, the corresponding physical P_{O_2} values were between 0.02 and 2 Pa, respectively. This was considered in the rise and drops of the O_2 measurements with the flies (see Discussion).

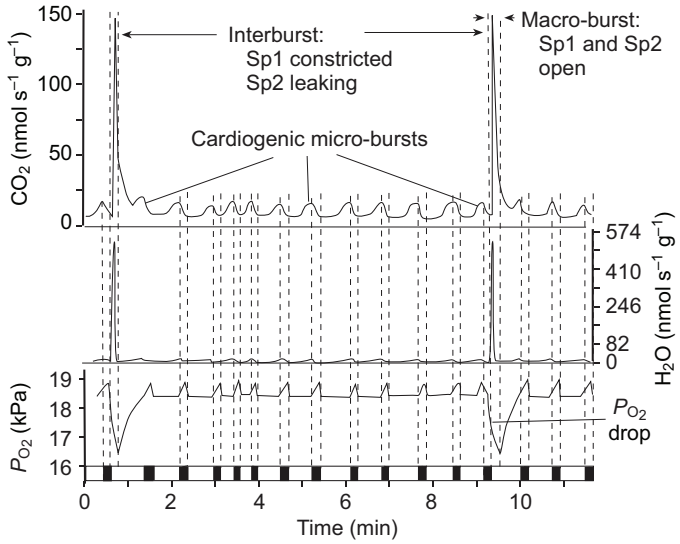

Fig. 12. Emission of CO_2, H_2O and periodicity of P_{O_2} in resting *C. vicina* (F7/07 at 20°C) depending on heartbeat reversals and spiracle leakage and open phases. Upper trace: periods with low amplitude cardiogenic CO_2 micro-bursts and high amplitude CO_2 macro-bursts during spiracle opening. Middle trace: H_2O peaks coincide with the CO_2 peaks. Lower trace: periodic oxygen peaks during backward pulse periods and P_{O_2} drops during spiracle opening in the scutellar air sac. Black bars, backward pulse periods.

Visualization and recording of spiracular valve action

For visualization of the valve action, the plates of ramified bristles of the peritrema, which hide the valve lips, were removed with surgical micro-scissors, paying special attention not to injure the membranous attachments of the valve lips to avoid bleeding. The movements of the thoracic spiracular valves were observed with a Macroscope (Leica M420: 35–70-fold magnification, Leica AG, CH-9435 Heerbruck) and recorded using the video and single-frame mode of a Eos D60 SLR camera (Canon, Otha-ku, Tokyo, Japan) or reflex measurements using a Nikon F2 camera (Nikon, Chiyoda-ku, Tokyo, Japan) with an integrated Si-photocell of 2.8×3.1 mm on the interchangeable SLR screen (Fig. 1B,C) with an external connection to the amplifying DC interface. The spiracles were illuminated with a light ring of white light-emitting diodes arranged around the front lens of the Macroscope. The surfaces of the valve lips are white with brownish, sclerotized areas. They have a higher reflectance than the shadowy tracheal lumen, which becomes exposed if the valves are open. On the basis of the video frames and photographs, the area of the spiracular valve opening was traced and calculated as the percentage of the maximal possible open area, using custom-made software.

Flow-through measurement of CO_2 emission and water loss

The CO_2 measurements were performed in a specimen chamber with controlled constant airflow (1000 ml min^{-1}) and adjustable pressure at controlled temperatures between 20 and 30°C. The volume of the specimen chamber was 20 ml and as small as possible (Fig. 1E) for recording at short response time for gas flow in the chamber. Response time was 1.4±0.2 s. The fly was glued at the mesoscutellum to a cannula, which at the same time served to fix the fly in position to the upper gum plug and connect the perforated air sac to the pressure sensor. A lateral port in the chamber allowed feeding and manipulation of a Styrofoam ball provided for foot contact in order to quieten the fly. The airflow stimulated prolonged running and grooming activities. Orientation of the head against the air stream appeased the flies noticeably. The chamber was connected directly to a CO_2/H_2O infrared gas analyser (LI-7000, LI-COR, Lincoln, NE, USA). Before entering the specimen chamber, the air passed a CO_2- and water-absorbing scrubber containing pellets of NaOH and the reference chamber. The pressure of the chamber was adjusted to a value between 10 and 50 Pa above ambient. The baseline of the CO_2/H_2O analyser and system was checked for drift after each experiment without a fly. The CO_2 output was calibrated between experimental runs in the absence of flies using a 50 ml syringe metering pump (Glenco, Houston, TX, USA), simulating the release of a constant volume of pure CO_2 gas in steps at different flow rates (0.1–10 µl s^{-1}).

Data acquisition

Data were continuously recorded on an Apple Powermac or Powerbook using a custom-made amplifier and a Powerlab 8-channel AD-Interface with

software (Chart 5.54: CB Sciences, Milford, MA, USA). The sampling rate was 200 Hz.

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Competing interests
The author declares no competing financial interests.

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Supplementary material
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