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RESEARCH ARTICLE

Flight-motor-driven respiratory airflow increases tracheal oxygen to nearly atmospheric level in blowflies (*Calliphora vicina*)

Lutz T. Wasserthal*

ABSTRACT

It is widely accepted that an efficient oxygen supply and removal of CO_2 in small flying insects are sufficiently performed by diffusion with open spiracles. This paper shows that in the tethered flying blowfly, gas exchange occurs by autoventilation and unidirectional airflow. The air is inspired through the mesothoracic spiracles (Sp1) during the downstroke of the wings and is expired through the metathoracic spiracles (Sp2) during the upstroke. This directed airflow through the thoracic tracheal system was documented by pre-atrial pressure measurements at the Sp1 and Sp2, revealing a sub-atmospheric mean pressure at the Sp1 and an over-atmospheric mean pressure at the Sp2. In the mesothoracic air sacs, the mean pressure is sub-atmospheric, conditioned by the only slightly open spiracles. In a split flow-through chamber experiment, the CO_2 released through the Sp2 confirmed this unidirectional respiratory gas flow, implicating an inner tracheal valve. In the thoracic tracheal system, the P_{O_2} during flight exceeds the high resting P_{O_2} by 1–2 kPa, reaching nearly atmospheric values. In the abdominal large air sacs, the P_{O_2} drops during flight, probably due to the accumulation of CO_2. Periodic heartbeat reversals continue during flight, with a higher period frequency than at rest, supporting the transport of CO_2 via the haemolymph towards the metathoracic tracheae and abdominal air sacs.

KEY WORDS: Autoventilation, Insect respiration, Tracheal pressure, Air sac, Tracheae, O_2 supply, Spiracles, Haemolymph, Gas exchange, CO_2 release, H_2O emission

INTRODUCTION

Flies are among the best fliers in the animal kingdom. They have indirect muscle fibres attached to the inner surface of the mesothoracic exoskeleton, which, by deformation, move the wings up and down. The high wingbeat frequency is maintained by stretch activation, based on the vibrational resonance of the thorax without synchronous nervous stimuli (Pringle, 1974). Among flies, Calliphoridae are powerful fliers. Vigorous flies can easily be obtained from the field and raised. Their size allows for micro-sensor access to the inner organs, such as the heart, accessory pulsatile organs and tracheal system, in contrast to the preferred fly model organism, *Drosophila*. In Calliphoridae, the structural basis and mechanics of the flight apparatus, its control and energetics have been investigated in detail (Miyan and Ewing, 1985; Nachtigall, 1985; Ennos, 1987; Wisser, 1988; Nalbach, 1989; Dickinson and Tu, 1997; Walker et al., 2014). There is a disproportion of thorough research on the flight mechanism in flies in contrast to the aspect of gas exchange during flight.

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Flight muscles consume more oxygen than do any other type of tissue. This applies especially to small insects, which expend relatively more energy compared with larger ones (Schmidt-Nielsen, 1997). An increase of blowfly metabolism during flight occurs by a factor of 100 (Davis and Fraenkel, 1940) and can be maintained for more than 30 min. This surpasses values measured in *Drosophila*, with an increase of CO_2 output that is 7.4 times higher during flight than at rest (Dickinson and Lighton, 1995) or of metabolic rate with respect to muscle mass by a factor of 8.5 during flight (Lehmann and Dickinson, 1997). Although it is generally accepted that flight is the most energy-absorbing activity, the mechanism of respiratory gas exchange in flying flies is not understood. Only a few papers address the question of how the high oxygen requirement during flight is achieved. In *Drosophila*, diffusion alone and adjustment of the spiracle opening have been suggested to provide adequate supply, sporadically supported by a hydraulic proboscis reflex (Lehmann and Heymann, 2005). The possible role of autoventilation by the flight apparatus in flies has been discussed and dismissed (Weis-Fogh, 1964). This scepticism was substantiated by the small contraction of the flight muscles of 1–2%, as well as the corresponding slight deformation of the thorax and tracheae (Boettiger, 1960). However, it was hypothesised that in contrast to a diffusive gas exchange, a convective gas exchange would provide a selective advantage, especially in small and flying insects, by reducing the respiratory water loss (Kestler, 1983, 1985).

Flies are sophisticated in most structural and functional regards, constituting the basis for their high flight performance. It was assumed that an ingenious mode of ventilatory gas exchange may be uncovered when using new and gentle analysing methods. Considering the indefatigable flight of blowflies, their oxygen supply should not run short during sustained flight. The present study tested the hypothesis that the small thoracic deformations by the asynchronous flight muscles in *Calliphora vicina* Robineau-Desvoidy 1830 also induce a ventilatory gas exchange, questioning the suggestion that diffusion alone provides sufficient gas exchange. A priority objective was the measurement of oxygen content in the tracheal air sacs using O_2-optodes concurrently with tracheal pressure and monitoring the wingbeat by photo-diodes. For analysing gas exchange, a technique similar to that successfully applied in the hawk moth *Manduca sexta* (Wasserthal, 2001) was adopted for this study to measure the tracheal pressure in front of the spiracles (pre-atrial) and in the dorsal air sacs, as well as the CO_2 release from specified spiracles using a split flow-through chamber. For comparison, the CO_2 and H_2O emissions of the entire fly were measured using flow-through respirometry with concurrent measurement of pressure or P_{O_2} in the scutellar air sacs. In addition, I analysed whether the periodic heartbeat reversals, which are basically involved in gas exchange at rest (Wasserthal, 1999, 2007, 2012, 2014), are continued during flight and whether they contribute significantly to gas exchange.

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RESULTS

Flight behaviour

The tethered flies showed different types of flight behaviour. The younger flies (1–4 weeks old) showed mainly intermittent volleys of several short flight phases, each lasting only few seconds or a fraction of a second with few wingbeats. Because of the high wingbeat frequency (range 132–159 Hz, mean 145±9.4 Hz, at 24°C, N=8), these short flights would be sufficient for flies to escape or change location. The volleys lasted up to 10 min. Long, continuous flight was performed more frequently by the older (1–6 months old) flies. The mean duration of the evaluated continuous flight was 117±79 s (N=7).

Dimensions of valve orifice of Sp1 and Sp2

The percentage of the aperture surface in relation to the maximum spiracle opening was calculated with custom-made software. The maximum possible spiracle opening was 0.234 mm^2 in the mesothoracic spiracles (Sp1) and 0.38 mm^2 in the metathoracic spiracles (Sp2). However, these wide apertures were rarely displayed and only for short moments. The thoracic spiracles often opened widest shortly at the initiation of a flight phase or after flight stop, with the consequence that the maximum P_{O_2} was attained at the end of flight or after flight had stopped. A typical opening during flight start was 0.036 mm^2 in Sp1 (Fig. 1A) and 0.14 mm^2 in Sp2 (Fig. 1B), representing a 3.9 times smaller aperture in the Sp1. The negative intratracheal pressure during flight was maintained with only slightly opened spiracles.

Relative tracheal pressure at the thoracic spiracles and in the air sacs

Tracheal pressure has a crucial influence on respiratory gas exchange. It reveals whether the gas exchange is performed by diffusion alone or through the support of convection. The pre-atrial pressure was measured at Sp1 and at Sp2 to evaluate a possible pressure gradient between anterior and posterior thoracic spiracles and to see whether heartbeat reversals have some influence on gas exchange during flight, just as documented during rest (Wasserthal, 2014). The pre-atrial pressure at the Sp1 and Sp2 was measured in different combinations: at a single spiracle, simultaneously at Sp1 and ipsilateral Sp2, or simultaneously at ipsilateral and contralateral spiracles (N=6). At all thoracic spiracles, the downstroke of the wings was correlated with a pressure decrease, while the upstroke was correlated with a pressure increase. During flight, the relative mean pressure at Sp1 on both sides was sub-atmospheric (−19±12 Pa, N=3) (Fig. 1A, Fig. 2). At Sp2, the mean pressure was over-atmospheric (5±3 Pa, N=3, Fig. 1B, Fig. 2). Positive pressure at Sp2 arose only if the flight was regular and stable. Irregular flight resulted in variable pressure above or at atmospheric levels (Fig. 1B). Unilateral wingbeat resulted in a one-sided pressure effect. If the left wing beat alone, the pressure decreased only at the ipsilateral spiracle and attained almost the same values as when the two wings were beating together (Fig. 3). There seems to be a side-bound pressure effect in the thorax and little pressure equalisation between the left and right tracheal system in the thorax.

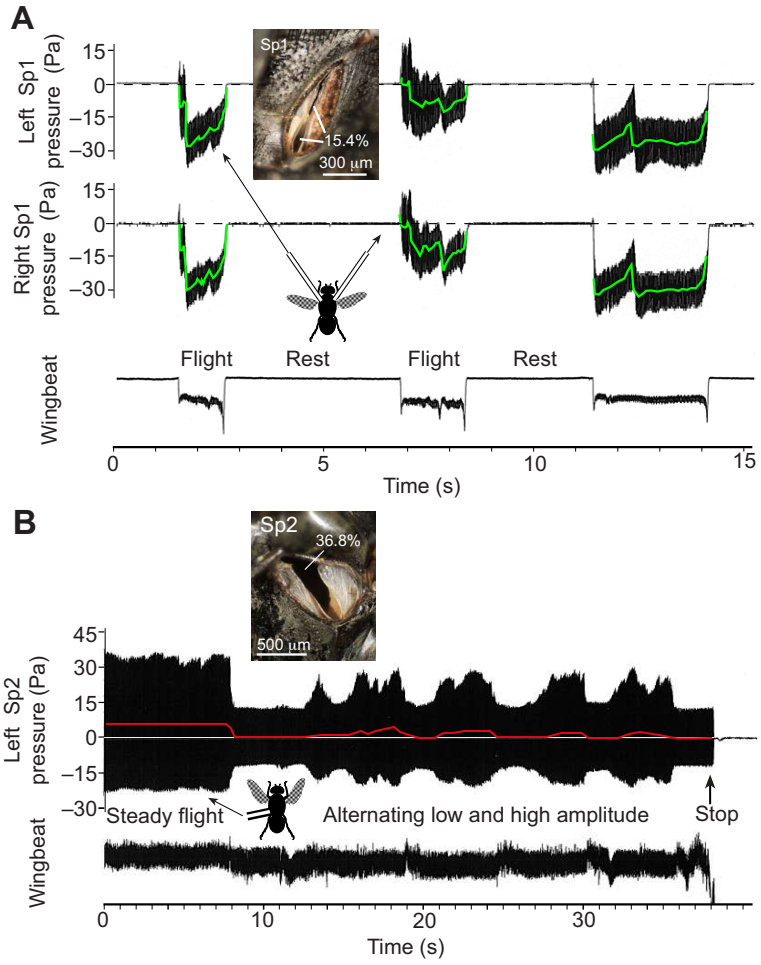

Fig. 1. Antidromic changes of relative pressure at the anterior and posterior thoracic spiracles during tethered flight. (A) At the mesothoracic spiracles (Sp1), pressure decreases to sub-atmospheric levels on both sides (female11*/2000); mean values, green curves. (B) At the metathoracic spiracle (Sp2), pressure increases to over-atmospheric levels (female12*/2000), 0=atmospheric pressure. The mean values (red curve) are over-atmospheric during steady flight and decrease towards zero (=atmospheric) during irregular flight. Insets: left Sp1 (A) and Sp2 (B) (filter bristles removed) showing the aperture condition at the onset of a short flight phase. The aperture of Sp1 exposes only 0.036 mm^2 (=15.4%) of the possible maximum opening; the aperture of Sp2 exposes only 0.14 mm^2 (=36.8%) of the possible maximum opening. *Captured wild specimen.

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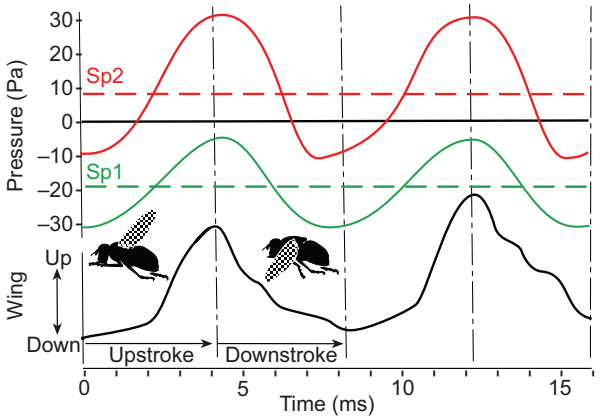

Fig. 2. Pre-atrial pressure differences between Sp1 and Sp2 during two wingbeat cycles. Data are for male15*/2000. At both tubed right spiracles, the pressure increases during upstroke and decreases during downstroke of the wings. The mean pressure at Sp1 (green dashed line) is negative, whereas the mean pressure at Sp2 (red dashed line) is positive. 0=atmospheric pressure. The wingbeat (black curve) is recorded by the shadow cast on a silicon photo-diode. Sampling rate: 40 kHz. *Captured wild specimen.

In the paired scutellar air sacs, side-specific pressure measurements were not analysed. The septum between neighbouring scutellar air sacs was always opened by the surgical intervention and the paired air sacs were connected in unity with the sensors. In the scutellar air sac, the mean tracheal pressure was always sub-atmospheric during flight: the relative air sac pressure decreased from the resting atmospheric values with open spiracles (=0 Pa) by −37.5±29.7 Pa (N=17, Fig. 4, Table 1). In this relative mean pressure decrease, the fluctuations of the positive and negative pressure periods in the tracheal system caused by heartbeat reversals, ranging between a mean of +8.8±13 and −8.1±17 Pa, are not relevant, as the spiracles during flight were more open than at rest, when most of the time they were only leaking (Wasserthal, 2014).

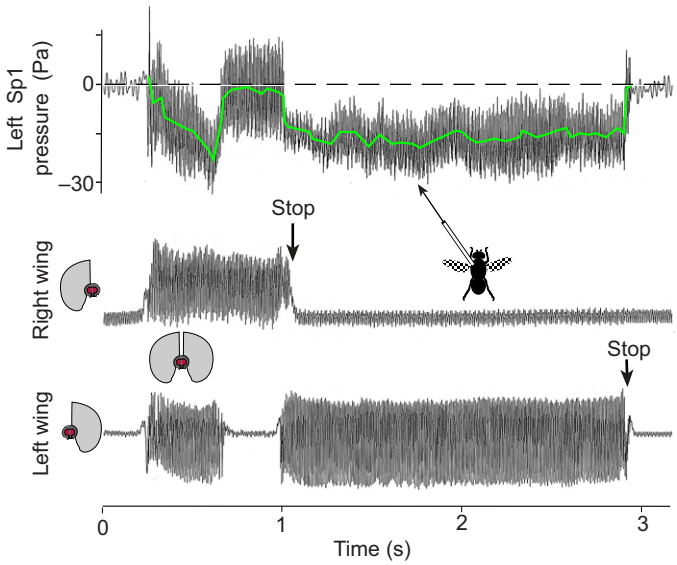

Fig. 3. Influence of ipsilateral and contralateral wingbeats on tracheal pressure at the left anterior spiracle (Sp1). Data are for female9*/2000. The mean pressure (green line) is sub-atmospheric when both wings or the ipsilateral wing is beating. If the contralateral (right) wing beats alone, the mean pressure at the left Sp1 is zero (=atmospheric). *Captured wild specimen.

Thoracic tracheal P_{O_2} is elevated and abdominal air sac P_{O_2} is reduced during flight

Resting flies maintained a high mean intratracheal P_{O_2} of 18.6±1.36 in the thorax, ventilated by cardiogenic haemolymph pressure changes (Wasserthal, 2014). It was unknown whether this oxygen concentration could be maintained or exceeded under high oxygen consumption during flight. P_{O_2} was measured in the scutellar or

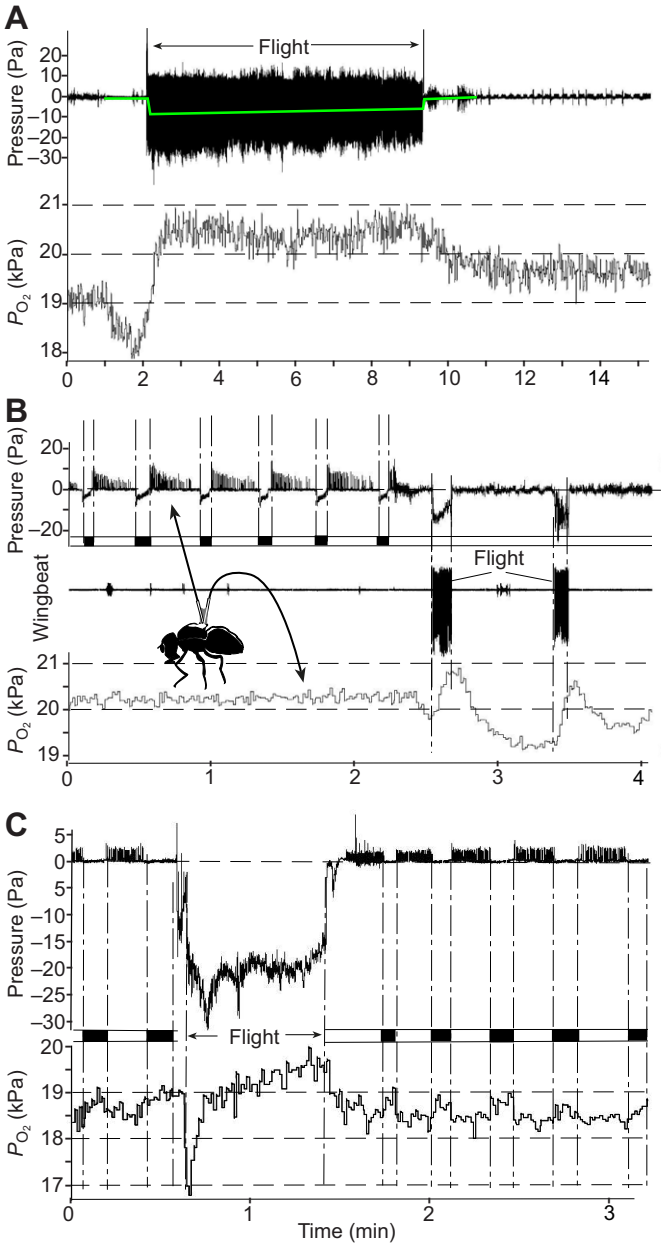

Fig. 4. P_{O_2} and relative intratracheal pressure in the scutellar air sac during rest and tethered flight. (A) During flight, the mean pressure (green curve) decreases to sub-atmospheric and the P_{O_2} increases to almost atmospheric (female14/2009, 38 days old). (B) After typical resting gas exchange characterised by periodic heartbeat reversals, the P_{O_2} exceeds the resting value of 20 kPa by 1 kPa during flight. After flight, the P_{O_2} decreases (female17*/2009). (C) Flight period between phases of rest with heartbeat reversals. Before or at the onset of flight, the P_{O_2} decreases rapidly and then rises 1 kPa over the resting values. After flight stops, the P_{O_2} decreases to the previous resting values (female2/2011, 137 days old). Black bars, backward periods of heartbeat, visualised by periodic pressure changes. *Captured wild specimen.

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Table 1. O_2 concentration during rest, pre-flight and flight measured in the mesoscutellar air sac simultaneously with intratracheal pressure

P_{O_2} (kPa)				
Rest	Pre-flight drop	Flight	ΔP_{O_2} during flight (kPa)	Intratracheal pressure (Pa)
18.6±1.4 (*N*=17)	16.7±1.97 (*N*=12)	19.5±1.27 (*N*=17)	1.05±0.37 (*N*=17)	–37.5±29.7 (*N*=17)

Values are means±s.d.; *N*, number of flies.
The intratracheal pressure value is the decrease in relative intratracheal pressure during flight.

abdominal air sacs using combined or separate pressure and O_2 sensors (Wasserthal, 2014). During short and steady flight, P_{O_2} increased in the scutellar air sacs. Before or at the initiation of flight, P_{O_2} decreased transiently by 1–2 kPa below the resting level (*t*-test, $P<0.005$, $N=12$, Fig. 4, Table 1). It then rapidly increased to 1–2 kPa above resting level, becoming almost atmospheric, with a mean P_{O_2} of 19.5±1.27 kPa (*t*-test, $P<0.02$, $N=17$).

In the abdomen, intratracheal P_{O_2} revealed an opposite effect. In the large abdominal air sacs during flight, P_{O_2} decreased from 17±0.94 kPa to 14±1.58 kPa (*t*-test, $P<0.002$, $N=6$, Fig. 5). It is assumed that CO_2 from the thoracic haemolymph was transferred into the air sacs before being released via the anterior three pairs of abdominal spiracles, which are connected to the air sacs. A similar P_{O_2} drop in the thoracic air sacs was previously found to be correlated with transient intratracheal CO_2 accumulation during spiracle opening in resting *C. vicina* (Wasserthal, 2014).

Temporal succession and intensity fluctuations of respiratory gas flows

The flight phases caused corresponding CO_2 and H_2O emissions. CO_2 emissions attained a mean value of 385±184 nmol s^{-1} g^{-1} and H_2O emissions attained a mean value of 216±110 nmol s^{-1} g^{-1} (Fig. 6A, Table 2). The flight phases were often initiated by an increase of CO_2 emissions without any visible activity (Fig. 6B, asterisk). These CO_2 peaks probably represent so-called macro-bursts, which are an effect of the spiracle opening during discontinuous gas exchange in resting insects (Wasserthal, 2014). The CO_2 emissions caused by intermittent flight phases replaced the CO_2 macro-bursts.

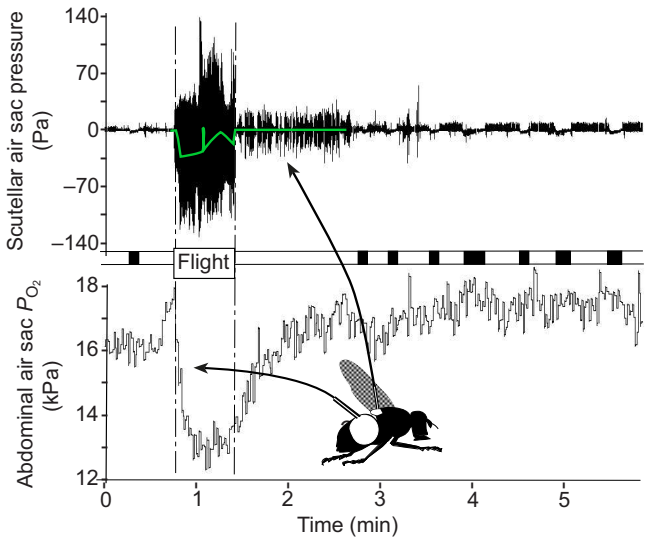

Fig. 5. P_{O_2} in the abdominal air sac and pressure in the scutellar air sac during tethered flight. Green, mean pressure (female14/2009, 38 days old). The P_{O_2} decrease is caused by an accumulation of CO_2 in the air sac. Black bars indicate periods of backward heartbeat.

CO_2 and H_2O release in the 20 ml flow-through chamber experiments with a flow rate of 1000 ml min^{-1} started with a response time of 1.4±0.2 s after onset of flight. While H_2O and CO_2 emissions increased during flight, tracheal P_{O_2} decreased at the beginning of flight (Fig. 7). In the course of the flight phases, tracheal P_{O_2} increased and attained its maximum at the end of the flight phase, sometimes even shortly after flight stopped. The preceding O_2 decrease is explained with a premature opening of the spiracles before flight onset. A periodic P_{O_2} drop in the scutellar air sac has already been described and interpreted as a consequence of the CO_2 transition from the haemolymph into the tracheal system before its release during the opening of the spiracles (Wasserthal, 2014).

The difference between the CO_2 emission levels during rest and tethered flight was not as high as one would have expected according to published data regarding the gain in metabolic rate of 148-fold from rest to full flight in hawk moths (Bartholomew and Casey, 1978). The CO_2 emissions during pauses between the flight phases were already rather high and the emissions during flight were only 3.3 times higher. After deep rest, the mean CO_2 emissions during flight were 33 times higher, or 81.4-fold when compared with the maximum values of CO_2 emissions at the beginning of the flight phases. The average values corresponded to a 15-fold increase of metabolic rate during flight in bumblebees (Ellington et al., 1990).

Water loss rose from 32.2±12 nmol s^{-1} g^{-1} at deep rest to 216±110 nmol s^{-1} g^{-1} during steady flight, which is a 6.7-fold increase. This rise is markedly below the 33-fold increase of the CO_2 output, from 11.7±9.4 nmol s^{-1} g^{-1} at rest to 385±184 nmol s^{-1} g^{-1} during flight (Table 2).

CO_2 release through the metathoracic and abdominal spiracles

As the pre-atrial pressure showed a decrement between Sp1 and Sp2, it was of interest to see whether inspiration and expiration were different at these spiracles. To examine the emissions through selected spiracles, the CO_2 emissions were measured using tubed flies in a split flow-through chamber, which allowed pairs of Sp1 or Sp2 to be connected separately with an interposed empty chamber to the CO_2-measuring device (infrared gas analyser, IRGA) (Figs 8, 9, 11). When the insect compartment was directly connected to the IRGA and the Sp2 tubes were deviated via the empty chamber into the scrubber, the emissions of the Sp1 and the abdominal spiracles were analysed together (Fig. 8A). The resulting CO_2 output was 24±10 nmol s^{-1} g^{-1} and followed on each flight period with a delay of 2–3 s ($N=4$). During the crosscheck experiment, the Sp2 was connected to the IRGA via the empty compartment, and 366±100 nmol s^{-1} g^{-1} of CO_2 was emitted ($N=4$, Fig. 8B).

To see whether and how much CO_2 is released by Sp1 alone, the Sp1 tubes were connected to the IRGA via the empty chamber (Fig. 9A). In this arrangement, a possible effect of all other spiracles was excluded. No CO_2 was emitted through Sp1 ($N=3$). In the crosscheck, Sp2 and all abdominal spiracles released CO_2 into the insect chamber without Sp1. The CO_2 emissions of Sp2 and of all abdominal spiracles were directed to the IRGA (Fig. 9B). The CO_2

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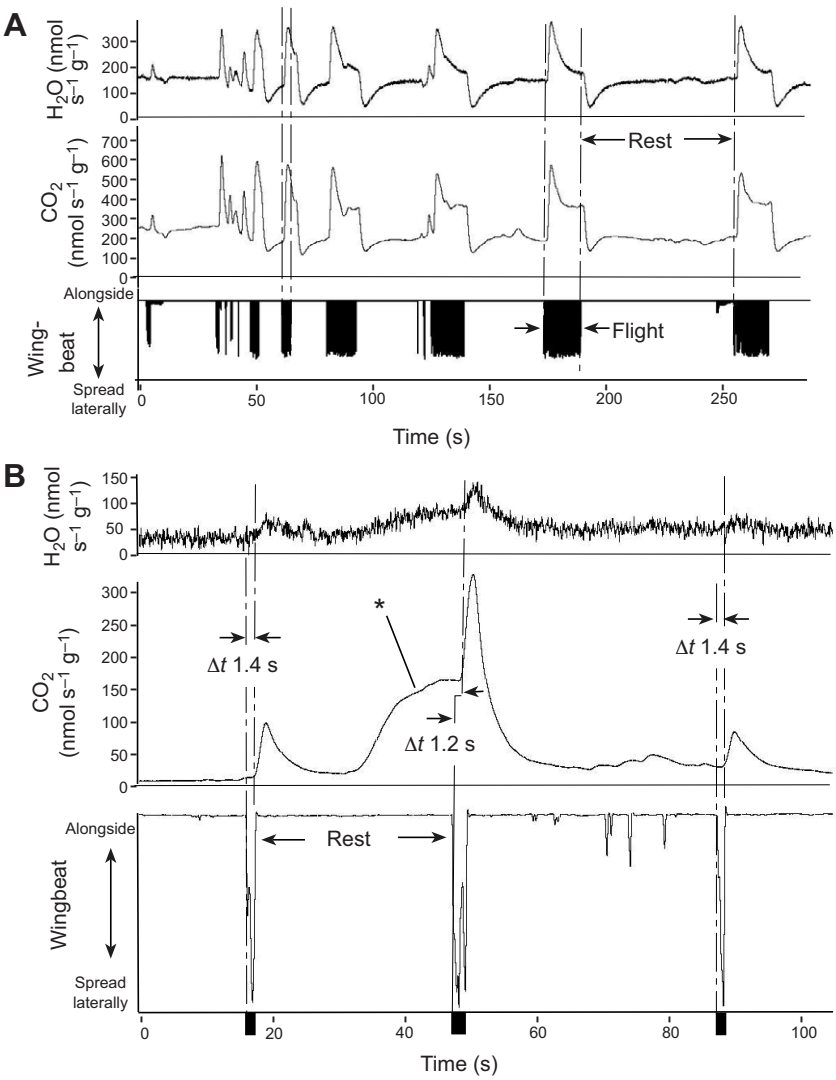

Fig. 6. Flow-through recordings of CO_2 and H_2O emissions during flight. Wing movements were recorded by the shadow cast on a Si photo-diode. (A) CO_2 and H_2O emissions start at a maximum level after onset of flight and settle down to a lower value during longer flight phases (female5/2014, 164 days old). (B) Three short flight events with higher temporal resolution. The response time of the CO_2 emissions is 1.4±0.2 s. One flight phase follows a CO_2 macro-burst (asterisk) (male15/2014, 4 days old).

level attained a similar value of 342±98 nmol s^{-1} g^{-1} (*N*=3), similar to the measurements of Sp2 output alone (Fig. 8B). The small fraction of CO_2 emissions in Fig. 9A must come from the abdominal spiracles because the contribution of Sp1 is zero (Fig. 8A). Because the small abdominal share of CO_2 emissions is not contained in the output of Sp2 alone (Fig. 8B), the bulk of CO_2 leaves from Sp2.

It was of interest to see whether the respiratory airflow was directed from Sp1 to Sp2 by an intratracheal valve mechanism, or whether it could be inverted by adjusting a pressure decrement between the compartments of the split chamber. While in the above experiments, the pressure in the two compartments of the split chamber was kept identical, in a second series, the pressure in the insect compartment was increased over that in the empty compartment with the discharging tubes of Sp1. A small pressure difference of +5 Pa in the insect compartment led to only a little, dampened increase of Sp1 emissions of below 24 nmol s^{-1} g^{-1} (*N*=3), as seen when comparing Fig. 9C with A. An increase of the pressure excess of +18 Pa in the insect compartment reversed the CO_2 emissions from the Sp2 to the anterior Sp1 (Fig. 9D). The amount (290–390 nmol s^{-1} g^{-1}, *N*=3) of the reversed CO_2 emissions was comparable with the unchanged airflow, but in contrast to the normal time response of 1–3 s, the artificially inverted airflow was delayed by 5–6 s after the initiation of flight. This experiment supports the existence of a tracheal valve, which prevents a reverse airflow, when the counter-pressure is moderate (Fig. 9C). This postulated valve seems to be overcharged by higher counter-pressure (Fig. 9D).

The role of the periodic heartbeat reversals in gas transport

Periodic heartbeat reversals have a significant influence on respiratory gas exchange in resting blowflies (Wasserthal, 2014). During flight, the thermistor measurements with heat-marking of the thoracic

Table 2. Emissions of H_2O and CO_2 during rest, tethered flight and interflight pauses of entire flies

	Interflight pause (nmol s^{-1} g^{-1})	Maximum (mostly initial) (nmol s^{-1} g^{-1})	Flight (nmol s^{-1} g^{-1})	Rest (nmol s^{-1} g^{-1})
H_2O (*N*=10)	101.7±69.1	391±192 Range: 200–1302	216±110	32.2±12
CO_2 (*N*=14)	116±63	953±517 Range: 537–1980	385±184	11.7±9.4

Values are means±s.d.; *N*, number of flies.

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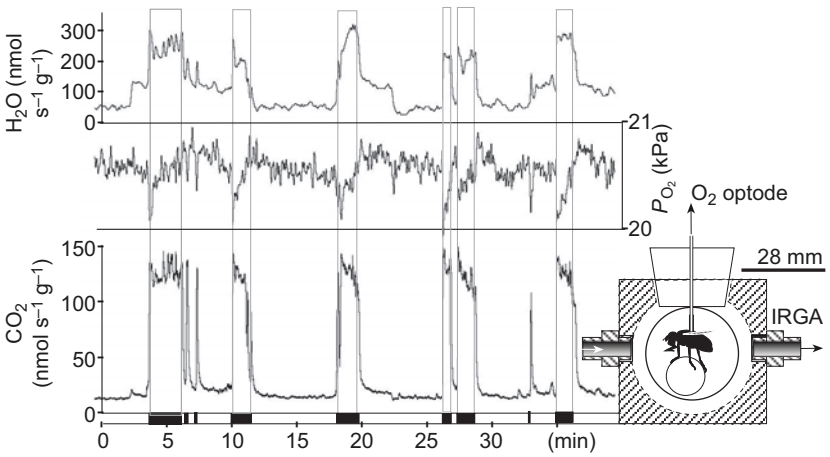

Fig. 7. Simultaneous recording of O_2 concentration in the scutellar air sac during respiratory flow-through measurement. Data are for female17/2014, 26 days old. Upper trace: H_2O emissions. Middle trace: P_{O_2} decreases at the beginning of the flight phase, then increases during most of the flight phase and attains the maximum level when flight stops. Lower trace: CO_2 emissions. Irrespective of the delay in response time, the level of H_2O and CO_2 emissions is congruent with the flight duration (black bars and boxed regions of traces). Inset shows an insect chamber for combined flow-through measurements and P_{O_2} measurement in the scutellar air sac. IRGA, infrared gas analyser.

haemolymph showed the typical periodic temperature increase above the abdominal heart characteristic for the backward pulses of the heart. During flight, no parallel conspicuous pressure effects were detected at Sp2 (Fig. 10). Owing to the increased thorax temperature of 27–31°C during flight, the heartbeat sequences appeared in a shorter succession (7±2 cycles min^{-1}, N=7), in contrast to the resting flies (1.53±0.61 cycles min^{-1} at 21°C, N=17; Wasserthal, 2014). The influence of the more frequent backward pulse periods supports a more efficient removal of the CO_2-charged haemolymph from the thorax into the abdomen, where it contacts the air sacs beside the Sp2 and the large abdominal air sacs. The rising CO_2 levels of the abdominal air sacs during flight also confirm this (Fig. 5).

DISCUSSION

Autoventilation and unidirectional air flow for increased oxygen supply during flight

Autoventilation in flying insects has been documented in larger insect species, such as locusts (Weis-Fogh, 1967; Miller, 1981). A unidirectional gas transport has been deduced from the segmental succession of spiracular openings. In large beetles, a passive inflow into the exposed anterior spiracles during incoming flow was called draught ventilation (Miller, 1981). In the hawk moth *M. sexta*, a unidirectional airflow with CO_2 emissions through the metathoracic spiracles represents a similar mechanism to that in the blowfly and lends itself to comparison. In the moth, the flight apparatus generates the suction force for the inspiration flow at the anterior spiracles as a result of inspiration prevention through the posterior thoracic spiracles (Wasserthal, 2001). During the downstroke, the volume of the thoracic air sacs increases, while the posterior thoracic spiracles are automatically enclosed in a pleural fold between the mesothorax and metathorax. During the upstroke, the air sac volume decreases and the moth expires through the exposed and open posterior spiracles. The mechanical coordination of spiracle opening and closing with the wingbeat cycle is possible because the wingbeat frequency of the moths is only 27±3 Hz and the longitudinal deformation of the thorax by the synchronous muscles is more enhanced.

In *Calliphora*, with a higher wingbeat frequency of 145±9.4 Hz performed by the asynchronous muscles, the spiracles act with a wingbeat-independent, less-frequent closing pattern. The metathorax with the posterior spiracles does not approach the mesothorax during downstroke and leaves the spiracle exposed. The pressure gradient between Sp1 and Sp2 of 20–34 Pa demonstrates that, during flight, a unidirectional airflow is produced, entering through the anterior spiracles (Sp1) and leaving through the posterior spiracles (Sp2).

How is the negative pressure at Sp1 and in the air sacs generated? The permanent sub-atmospheric pressure in the scutellar air sac during flight is based on the sub-atmospheric pressure in the haemocoel (Wasserthal, 2012). During flight, the increased consumption of O_2 might further contribute to the reduction of tracheal pressure as, because of the haemolymph buffering capacity, not all CO_2 is released immediately into the tracheal system to compensate for the loss of O_2 (Chown et al., 2006). In addition, the strict correlation of the pressure decrease with each downstroke of the wings suggests a mechanical effect, which is produced by the volume changes of the deforming thorax. The resulting pressure difference causes a unidirectional airflow by autoventilation. This conclusion is substantiated by the split-chamber flow-through experiments, which show the bulk of CO_2

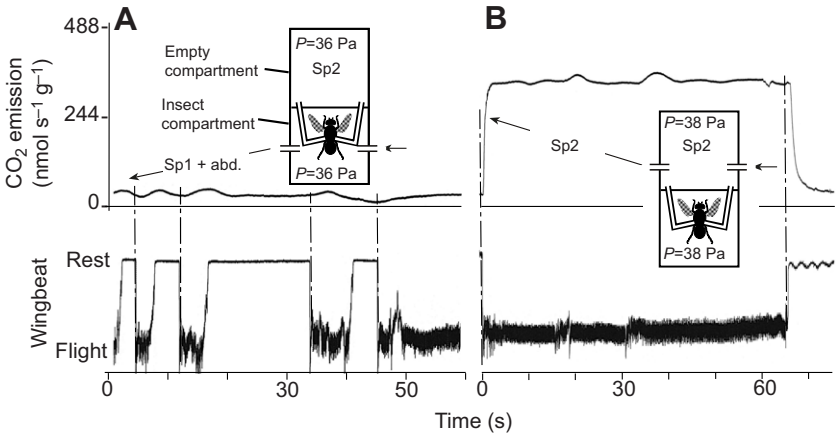

Fig. 8. CO_2 emissions in a flying blowfly with tubed Sp2 in a split-chamber flow-through experiment. Data are for female12*/2000. (A) The insect compartment with the first thoracic (Sp1) and all abdominal spiracles is connected directly to the IRGA, while the tubed posterior thoracic spiracles (Sp2) open into the empty compartment, which is connected to the CO_2 scrubber. Only a small amount of CO_2 is emitted during each flight phase from the Sp1+abdominal (abd.) spiracles. (B) In the crosscheck, only the Sp2 tubes are connected to the IRGA via the empty compartment. The bulk of the CO_2 is emitted from the Sp2. *Captured wild specimen.

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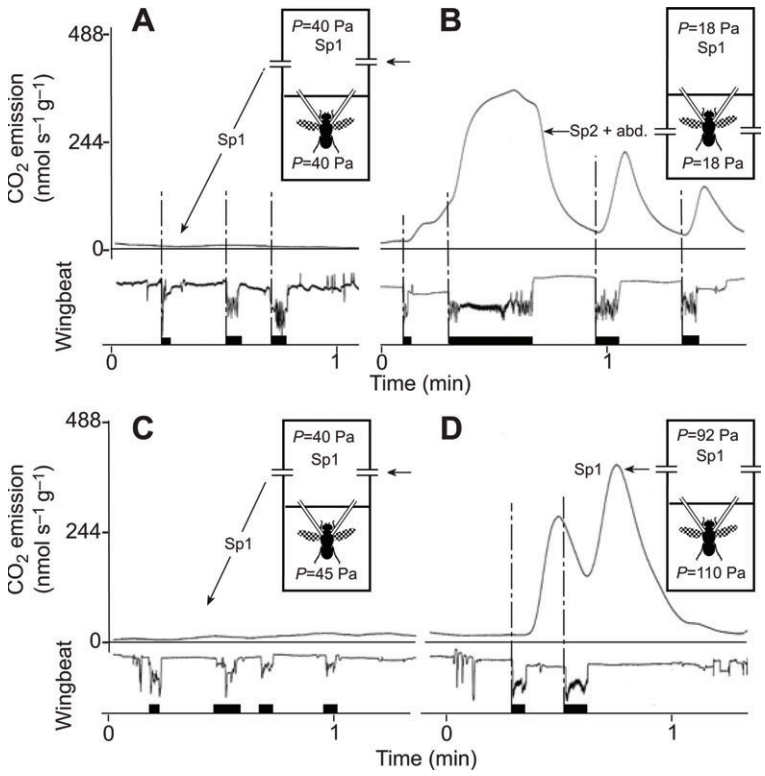

Fig. 9. CO_2 emissions in a flying blowfly with tubed Sp1 in a split-chamber flow-through experiment. Data are for female12*/2000. (A) The tubed first thoracic spiracles (Sp1) are connected to the IRGA via the empty compartment, while the insect compartment with the posterior thoracic and abdominal spiracles (Sp2+abd.) is connected to the CO_2 scrubber. No CO_2 is emitted from the Sp1. (B) In the crosscheck, the insect compartment is connected to the IRGA. The Sp1 are connected to the scrubber. All CO_2 comes from the Sp2 and abdominal spiracles. The delay between the onset of wingbeat and detection by the IRGA is 1–2 s. (C,D) Split-chamber experiments with artificially increased pressure in the insect compartment (male15*/2000). The tubed first thoracic spiracles (Sp1) are connected to the IRGA, while the insect compartment with the posterior thoracic and abdominal spiracles (Sp2+abd.) is connected to the CO_2 scrubber. (C) Although the pressure is 5 Pa higher in the insect compartment than in the empty compartment, almost no CO_2 is emitted during the flight phases from the Sp1. (D) After increasing the pressure in the insect compartment by 18 Pa over that of the empty compartment with the discharging Sp1 tubes, the tracheal airflow is now reversed and CO_2 is emitted from the Sp1. The delay between the onset of wingbeat and the detection of CO_2 emissions by the IRGA is 5–6 s. Black bars correspond to the flight phases. *Captured wild specimen.

release through the Sp2 and, to a smaller extent, from the abdominal spiracles (Figs 8, 9).

Diffusion alone in small insects was regarded as sufficient for respiratory gas exchange during flight (Weis-Fogh, 1964; Miller, 1974; Lehmann and Heymann, 2005). The possibility of gas exchange by ventilation due to thoracic distortions in small insects has been disclaimed because the deformations of the thorax by the indirect flight muscles are very small. The contraction of the indirect flight muscles in *Sarcophaga* blowflies is only 1–2% of its resting length (Boettiger, 1960) or 2–5% of the strain amplitude in *Drosophila virilis* (Chan and Dickinson, 1996). Moreover, in *Drosophila*, it was argued that in addition to the small thoracic distortion in these small tracheae, autoventilation and tracheal ventilation due to the Bernoulli effect are greatly attenuated by the low Reynolds number for tracheal airflow (Lehmann, 2001); Heymann and Lehmann (2006) state: 'Although tethered flying *Drosophila* sporadically employ ventilation to manipulate tracheal gas flow, diffusion is still assumed to be the main type of respiration in fruit flies and diffusive theory may be applied'. These authors concluded that their 'present results provide direct evidence for the general assumption in respiratory research that the tracheal development of a simple diffusion-based system matches the respiratory needs at maximum metabolic activity of the animal' (Heymann and Lehmann, 2006).

However, it has been shown that distortion of the thorax in *Calliphora* results in upward and outward movement during the downstroke: 'The dorsolongitudinal muscles provide increased scutal arching, localised at the scuto-scutellar border and outward splaying of the tergal fissure' (Ennos, 1987). In video sequences of *C. vicina*, the scutal arching during downstroke is 60–96 µm of a 5 mm high thorax, confirming the small, approximately 1–2%, vertical distortion (L.T.W., unpublished).

In *C. vicina* fixed dorsally at the meso-scutum, the scutellum moves downwards during downstroke and the antero-ventral thorax prolongates by 3.3–4.7%. This distortion is sufficient to enlarge the thoracic volume during the downstroke, increasing the volume of

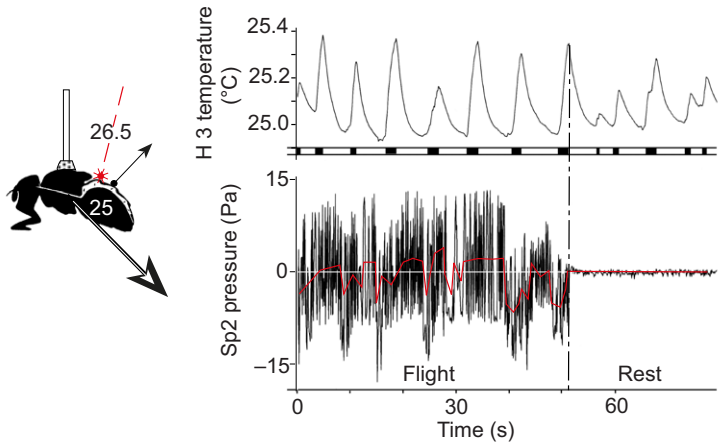

Fig. 10. Thermistor records of periodic heartbeat reversals continuing during flight. The temperature peaks during backward pulses (black bars) were measured using a thermistor glued on the 3rd tergite (H 3 temperature). The procedure is done by heat-marking of the haemolymph by the flight muscles aided by a laser beam, which is projected onto the 2nd abdominal tergite. The number of backward heartbeat periods is 9 min^{-1}. There is no clear effect of heartbeat period on the course of the pressure curve (male 8/2007, 57 days old).

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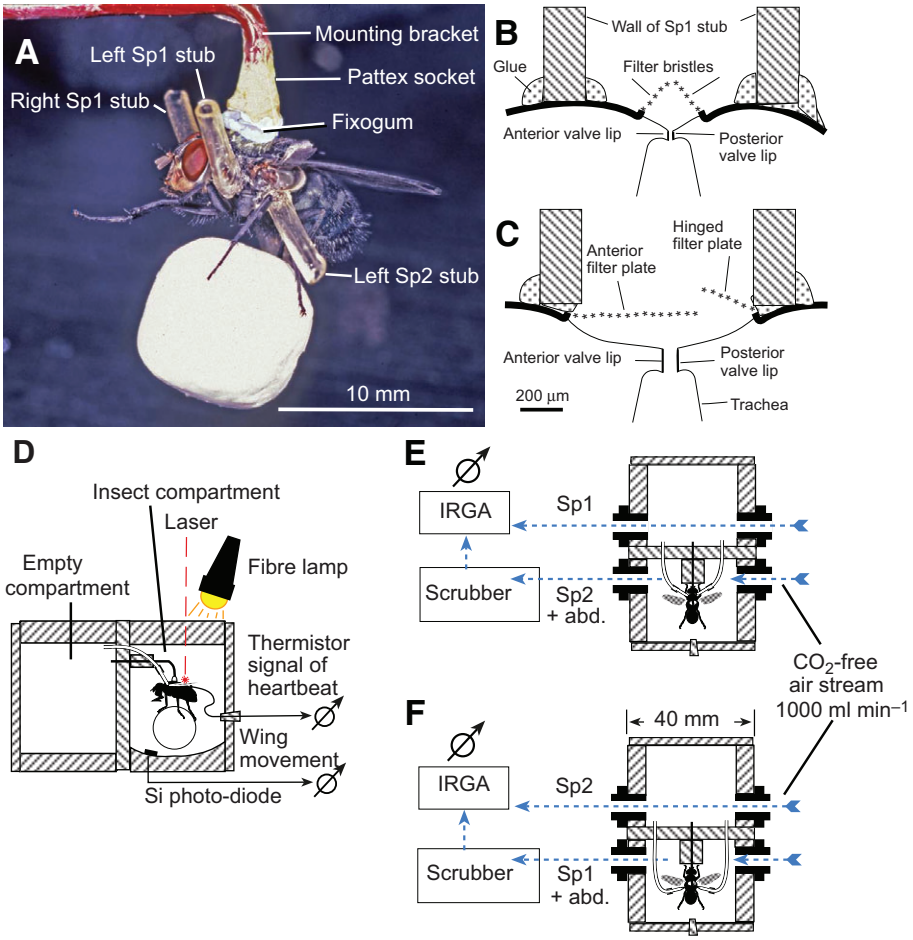

Fig. 11. Split Plexiglas chamber for selective measurement of CO_2 emissions from specified spiracles of the blowfly. (A) Tethered fly prepared with four polyethylene stubs glued at the mesothoracic (Sp1) and metathoracic (Sp2) spiracles on both sides. (B) Schematic longitudinal section of Sp1. (C) Schematic longitudinal section of Sp2. (D) Lateral view of the split chamber. The floor of the specimen compartment is equipped with a silicon photo-diode for recording the light–dark effect of the wing shadow. A thermistor glued on the abdomen serves to record the heartbeat. A lateral port allows for manipulations (provisioning or removal of the Styrofoam ball). The volume of the insect compartment and of the empty compartment is 18 ml each. (E) The tubed anterior spiracles (Sp1) are connected to the IRGA via the empty compartment. (F) The tubed posterior spiracles (Sp2) are connected to the IRGA via the empty compartment. Devices for regulation and measurement of air speed and chamber pressure have been omitted.

the scutal air sac and the tracheae between the longitudinal and vertical muscle bundles (L.T.W., unpublished). When the air sacs expand, they produce a negative pressure pulse. The high-frequency summation of the pulses (145±9.4 Hz) leads to the mean sub-atmospheric pressure at the Sp1 and in the mesothoracic tracheal system. The suction at the Sp1 is the precondition for the uniform inflow of fresh air and the rise from a mean resting P_{O_2} of 18.6 to 19.5 kPa oxygen supply during flight in the scutellar air sac. This relatively high O_2 concentration corresponds to the high P_{O_2} in flying hawk moths (Komai, 1998) and bumblebees (Komai, 2001). However, the question remains as to why the mean tracheal pressure continues to be sub-atmospheric and no CO_2 is released through Sp1 during the upstroke. The existence of a back-pressure valve behind Sp1 is therefore postulated. The experiments with the artificially imposed higher pressure on the insect compartment of the split flow-through chamber with the intention to reverse the respiratory air stream from Sp2 to Sp1 are in favour of the occurrence of a valve that is, however, only effective as long as the counter-pressure is moderate and not higher than 5 Pa; it did not resist higher, probably un-physiological pressure.

A comparison of the outer morphology of Sp1 and Sp2 reflects the different functions (tasks) of these spiracles. Sp1 has a 3.9-fold narrower aperture than Sp2 when, for example, Sp1 is 15% open and Sp2 is 37% open (Fig. 1). Sp1 has a fixed roof of filter bristles, whereas the hinged posterior filter plate of Sp2, which is pressed passively outwards by a strong expiratory airflow, supports the expiratory function of Sp2. The negative intratracheal pressure in the mesothorax during flight is maintained by only a slight opening of the spiracular valves. It is suggested that a wide opening of the valves causes a pressure equalisation with the atmosphere, resulting in a breath. The convective oxygen gain would, however, diminish when the spiracles remain wide open for a longer time, preventing the re-establishment of a negative tracheal pressure.

Proboscis extension for tracheal ventilation

Proboscis movements in *Drosophila* during flight have been reported to represent a sporadic type of tracheal ventilation (Lehmann and Heymann, 2005). The flight-associated proboscis movements may be a taxon-specific trait. In *C. vicina*, proboscis pulsations were not observed during flight. The ventilation effect by extension and retraction of the proboscis has, however, been recorded in resting *C. vicina* and the hoverfly *Eristalis tenax* (L.T.W., unpublished). Here, tracheal pressure pulses were correlated with the dabbing of water or honey solution. During extension, the haemocoel of the proboscis is filled with haemolymph and its volume is compensated for by the expanding cephalic air sacs, causing inhalation. At rest and only rarely during flight, the flies regurgitated and slowly resucked their crop contents without dabbing and pressure pulses.

Bouts of droplet extrusion were also performed by resting calliphorid flies under high ambient temperatures in the field (L.T.W., unpublished). Proboscis extension and fluid exposition were performed under high ambient temperature (above 28°C) and interpreted as a mechanism for evaporative cooling in the hawk moth *Pholus achemon* (Adams and Heath, 1964) and in honeybees (Heinrich, 1979, 1980). *Calliphora vicina* often discharged fluid from the crop during and after preparation for the experiment. This

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is interpreted as a stress reaction, possibly relieving the body weight for escape flight. Before attributing the proboscis extrusion to ventilation in flying *Drosophila* (Lehmann and Heymann, 2005), the authors stated that the proboscis extensions were often correlated with water emission spikes during the initial stage of flight, soon after the fly had been placed into the respiratory chamber: 'Water spikes only occur during flight and are often correlated with the extension of the fly's proboscis' (Lehmann et al. 2000).

It is questionable whether proboscis extension is a typical ventilation procedure during flight in flies. It is probable that it exposes the humidified tongue for evaporative cooling or when suffering from stress. The CO_2 emissions during proboscis retraction would then be a side effect as is the case during dabbing at rest (L.T.W., unpublished).

Conclusions

Flying blowflies generate a unidirectional ventilatory airflow. This was concluded from the pressure difference between Sp1 and Sp2 and was confirmed by the selective CO_2 release through the posterior spiracles (Sp2 and abdominal spiracles). The gas exchange is performed by the flight apparatus, deforming the thoracic air sacs. This autoventilation gas exchange – despite the small deformations of the thorax by only 1–2% – raises the oxygen concentration within the thoracic air sacs to nearly ambient levels during full flight. These findings contradict the opinion that respiratory gas exchange during flight in small insects relies on diffusion alone. Instead, they confirm the hypothesis – based on a theoretical model – 'that replacement of diffusive gas exchange by convective gas exchange should be favoured in evolution of small animals, whenever it is possible' to reduce respiratory water loss (Kestler, 1985). Experiments with pressure reversal in the tracheal system suggest the existence of an intratracheal valve mechanism. An X-ray tomographic analysis of the tracheal system needs to be performed to answer this question (L.T.W. and P. Cloetens, in preparation).

MATERIALS AND METHODS

Animals

Blowflies (*C. vicina*) were captured from the field. They were examined immediately, or the F1 offspring of vigorous females were bred and the adults were used after the fourth day at the earliest point after eclosion. Some flies were kept alive in a flight cage for up to 6 months, hibernating in a cool winter garden. For presentation of the results, the data for specimens with different ages (mostly between 5 days and 2 months) were pooled. There was no obvious fundamental difference in the behaviour and the physiological reactions in flies of different ages. However, the older flies were much more appetent to fly longer continuously. The use of the wild *C. vicina* and F1 generation guaranteed powerful fliers. The procedure to engage wild specimens and only the F1 generation avoided inbreeding and was a consequence of my experience that flies from commercial or homogeneous laboratory stocks were short-lived and incapable of flying persistently. The mean mass and s.d. of the flies was 84±18 mg. Their mass changed by 18% during the experimental runs, depending on the fluctuating intestine content. Several times a day, the flies were given a droplet of honey–water solution on the Styrofoam running ball. Flies were anesthetised with CO_2 gas only for fixation and surgical treatment.

Measurement of spiracular valve openings

The spiracular valves were recorded by video sequences during rest and flight, as described in Wasserthal (2014). The imported videos were then analysed using Autodesk Maya 3 (111 McInnis Parkway, San Rafael, CA, USA). The contours of the valve lips around the aperture were traced in an upper layer, and the positional changes were depicted along the time axis with the 'Hypergraph' tool (Autodesk).

Pressure measurements at the thoracic spiracles

For measurement of the pre-atrial pressure, the mesothoracic spiracles (Sp1) and metathoracic spiracles (Sp2) were connected to polyethylene tubes (outer diameter 1.2 mm, inner diameter 0.8 mm, Fig. 11A–C). The plastic tubes or stubs were glued with Pattex (Henkel, Düsseldorf, Germany) and sealed in front of the spiracular peritrema with Fixogum rubber cement (Marabu, Tamm, Germany), without injuring the atrium and valves. These pre-atrial measurements recorded the atrial pressure conditions, which, in the resting flies, essentially corresponded to the intratracheal pressure in the scutellar air sacs when the spiracular valves were open (Wasserthal, 2014). The pressure measurements at the spiracles were not unproblematic because the tight connection of the spiracles with the sensor blocked the gas exchange with the ambient air. The measuring device was a closed system with a limited gas volume of 60 µl on each side. If both spiracles of the same thoracic segment were blocked, dyspnoea occurred. This became visible in the form of short and intermittent flight phases or irregular flight. The air volume had to be replenished by repeated opening of the connecting tubes. If only one spiracle of the same segment was connected to the pressure sensor, gas exchange was less restricted and flight was more sustained. The restricted gas exchange on one side was probably not fully compensated for via the open contralateral spiracle. This is suggested by the fact that the contralateral wing movement did not have the same pressure-decreasing effect at the Sp1 as the ipsilateral wing movements (Fig. 3).

For O_2 and pressure measurements, the flies were fixed at the mesoscutellum or abdominal tergite 2. For CO_2 and H_2O flow-through experiments, the flies were glued to the mesonotum. In all experiments, a combination of Pattex with a layer of Fixogum was used. The Fixogum functioned like an elastic cushion, which was necessary to avoid impeding the thoracic deformations caused by the indirect flight muscles. Otherwise, the flies refused to fly. There were no visual movement patterns used for flight activation. Flight started spontaneously or as a reflex action when the support, a Styrofoam running ball, was withdrawn from the legs.

Measurement of tracheal air sac pressure and heartbeat

For measurement of the tracheal pressure inside the body, the dorsal cuticle with the underlying air sacs was perforated and connected to a bi-tubed plastic cone using a mixture of Pattex and Fixogum, which allowed the fly to be handled and tethered in the setup, while being simultaneously connected to the pressure sensor (Sensym SCXL 004 DN, Sensor-techniques, Puchheim, Germany) and to the O_2 optode. The dead space of the pressure 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 and tables. The response time was 7–10 ms and the time constant was 30 ms.

Heartbeat was measured by thermistors utilising the thermal effect of the metabolic heat of the flight muscles or by artificially warming the haemolymph of the thorax and anterior abdomen by a laser beam, as previously described (N=7, F1 offspring, 6–57 days old; Wasserthal, 2012).

Measurement of tracheal P_{O_2}

Measurement of the P_{O_2} was performed using fibre-optic optodes (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, and arranged beside the air pressure tube in the bi-tubed adapter cone (Wasserthal, 2014). The measurements were run under controlled ambient temperature, between 20 and 24°C. 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 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 for continuous use over several weeks without significant reduction in sensitivity and only a slow, gradual loss in response time. For further details, see Wasserthal (2014).

Flow-through measurement of CO_2 and H_2O emissions

CO_2 and H_2O emissions of flies without spiracle tubes were measured in a 20 ml volume flow-through chamber. The flies were glued by the meso-notum to a bracket. The chamber was connected directly to a CO_2/H_2O

IRGA (LI-7000, LI-COR, Lincoln, NE, USA). Before entering the reference chamber and the specimen chamber, the air passed a CO_2- and water-absorbing scrubber containing pellets of NaOH. The airflow was a constant 1000 ml min^{-1} at controlled temperatures of between 20 and 24°C and controlled pressure. This was slightly over-atmospheric (ΔP=10–100 Pa) to avoid unwanted gas inflow by tiny leaks. In a number of experiments, the flies were fixed at the scutellum by a cannula passing through the upper plug and connecting the scutellar air sac with the O_2 optode (N=3) or pressure sensor (N=6), as described earlier (Wasserthal, 2014; Fig. 7).

Split-chamber experiment

For measurement of the CO_2 release from selected spiracles, the insects were transferred into the 18 ml insect compartment of a split flow-through chamber as described previously (fig. 1 in Wasserthal, 2001). The 4 mm long tube stubs of the spiracles were extended by 24–32 mm long polyethylene tubes connected via sleeves and canalised into the 18 ml empty compartment of the split chamber (Figs 8, 9, 11). The respiratory air stream of both Sp1 and both Sp2 could thus be separately directed to the gas analyser, while the insect compartment was connected to the scrubber, or vice versa. By exemption, the effects of Sp1, Sp2 and abdominal spiracles could be derived. The pressure inside the compartments of the split chamber was measured and adjusted to identical values. For checking the possible occurrence of an internal tracheal valve, an artificial pressure decrement between the compartments was produced in order to test the possibility of a flow reversal. In the split-chamber experiments, the CO_2-free air was humidified before passing through the chamber compartments.

Recording of the wingbeat

The wingbeat was recorded by measuring the changes in brightness caused by the upstroke and downstroke, interrupting a constant light beam projected on a Si photo-diode. The wingbeat frequency was determined by optical freezing of the wing position by stroboscopic flashes or by recording the pressure pulses with a high sampling rate of 1–40 kHz.

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Competing interests

The author declares no competing or financial interests.

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References

Adams, P. A. and Heath, J. E. (1964). An evaporative cooling mechanism in *Pholus achemon* (Sphingidae). *J. Res. Lepid.* **3**, 69-72.
Bartholomew, G. A. and Casey, T. M. (1978). Oxygen consumption of moths during rest, pre-flight warm up and flight in relation to body size and wing morphology. *J. Exp. Biol.* **76**, 11-25.
Boettiger, E. G. (1960). Insect flight muscles and their basic physiology. *Annu. Rev. Entomol.* **5**, 1-16.
Chan, W. P. and Dickinson, M. H. T. (1996). In vivo length oscillations of indirect flight muscles in the fruit fly *Drosophila virilis*. *J. Exp. Biol* **199**, 2767-2774.
Chown, S. L., Gibbs, A. G., Hetz, S. K., Klok, C. J., Lighton, J., R. B. and Marais, E. (2006). Discontinuous gas exchange in Insects: a clarification of hypotheses and approaches. *Physiol. Biochem. Zool.* **79**, 333-343.
Davis, R. A. and Fraenkel, G. (1940). The oxygen consumption of flies during flight. *J. Exp. Biol.* **17**, 402-407.
Dickinson, M. H. and Lighton, J. R. (1995). Muscle efficiency and elastic storage in the flight motor of *Drosophila*. *Science* **268**, 87-90.
Dickinson, M. H. and Tu, M. S. (1997). The function of dipteran flight muscle. *Comp. Biochem. Physiol. A Physiol.* **116**, 223-238.
Ellington, C. P., Machin, K. E. and Casey, T. M. (1990). Oxygen consumption of bumblebees in forward flight. *Nature* **347**, 472-473.
Ennos, A. R. (1987). A comparative study of the flight mechanism of Diptera. *J. Exp. Biol.* **127**, 355-372.
Heinrich, B. (1979). Keeping a cool head: honeybee thermoregulation. *Science* **205**, 1269-1271.
Heinrich, B. (1980). Mechanisms of body-temperature regulation in honyebees, *Apis mellifera*. I. Regulation of head temperature. *J. Exp. Biol.* **85**, 61-72.
Heymann, N. and Lehmann, F.-O. (2006). The significance of spiracle conductance and spatial arrangement for flight muscle function and aerodynamic performance in flying *Drosophila*. *J. Exp. Biol.* **209**, 1662-1677.
Kestler, P. (1983). Weshalb gerade ganz kleine Insekten im Flug einen Gasaustausch durch Diffusion verhindern müssen. In *BIONA-report*, Vol. 2 (ed. W. Nachtigall), pp. 11-20. Stuttgart: Akad. Wiss. Mainz, Fischer.
Kestler, P. (1985). Respiration and respiratory water loss. In *Environmental Physiology and Biochemistry of Insects* (ed. K. H. Hoffmann), pp. 137-183. Berlin: Springer.
Komai, Y. (1998). Augmented respiration in a flying insect. *J. Exp. Biol.* **201**, 2359-2366.
Komai, Y. (2001). Direct measurement of oxygen partial pressure in a flying bumblebee. *J. Exp. Biol.* **204**, 2999-3007.
Lehmann, F.-O. (2001). Matching spiracle opening to metabolic need during flight in *Drosophila*. *Science* **294**, 1926-1929.
Lehmann, F.-O. and Dickinson, M. H. (1997). The changes in power requirements and muscle efficiency during elevated force production in the fruit fly *Drosophila melanogaster*. *J. Exp. Biol.* **200**, 1133-1143.
Lehmann, F.-O. and Heymann, N. (2005). Unconventional mechanisms control cyclic respiratory gas release in flying *Drosophila*. *J. Exp. Biol.* **208**, 3645-3654.
Lehmann, F.-O., Dickinson, M. H. and Staunton, J. (2000). The scaling of carbon dioxide release and respiratory water loss in flying fruit flies (*Drosophila* spp.). *J. Exp. Biol.* **203**, 1613-1624.
Miller, P. L. (1974). Respiration-aerial gas transport. In *The Physiology of Insecta*, Vol. 6 (ed. M. Rockstein), pp. 345-402. New York: Academic Press.
Miller, P. L. (1981). Ventilation in active and inactive insects. In *Locomotion and Energetics in Arthropods* (ed. C. F. Herreid and C. R. Fourtner), pp. 367-390. New York: Plenum Press.
Miyan, J. A. and Ewing, A. W. (1985). How Diptera move their wings: a re-examination of the wing base articulation and muscle systems concerned with flight. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **311**, 271-302.
Nachtigall, W. (1985). *Calliphora* as a model system for analysing insect flight. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Vol. 5(15) (ed. G. A. Kerkut and L. I. Gilbert), pp. 501-605.
Nalbach, G. (1989). The gear change mechanism of the blowfly (*Calliphora erythrocephala*) in tethered flight. *J. Comp. Physiol. A* **165**, 321-331.
Pringle, J. W. S. (1974). Locomotion: Flight. In *The Physiology of Insecta*, Vol. 3(7) (ed. H. Rockstein), pp. 433-476.
Schmidt-Nielsen, K. (1997). *Animal Physiology: Adaptation and Development*, p. 613. Cambridge: Cambridge University Press.
Walker, S. M., Schwyn, D. A., Mokso, R., Wicklein, M., Müller, T., Doube, M., Stampanoni, M., Krapp, H. G. and Taylor, G. K. (2014). In vivo time-resolved microtomography reveals the mechanics of the blowfly flight motor. *PLOS Biol.* **12**, e1001823.
Wasserthal, L. T. (1999). Functional morphology of the heart and of a new cephalic pulsatile organ in the blowfly *Calliphora vicina* (Diptera: Calliphoridae) and their roles in hemolymph transport and tracheal ventilation. *Int. J. Insect Morphol. Embryol.* **28**, 111-129.
Wasserthal, L. T. (2001). Flight-motor-driven respiratory air flow in the hawkmoth *Manduca sexta*. *J. Exp. Biol.* **204**, 2209-2220.
Wasserthal, L. T. (2007). *Drosophila* flies combine periodic heartbeat reversal with a circulation in the anterior body mediated by a newly discovered anterior pair of ostial valves and 'venous' channels. *J. Exp. Biol.* **210**, 3707-3719.
Wasserthal, L. T. (2012). Influence of periodic heartbeat reversal and abdominal movements on hemocoelic and tracheal pressure in resting blowflies *Calliphora vicina*. *J. Exp. Biol.* **215**, 362-373.
Wasserthal, L. T. (2014). Periodic heartbeat reversals cause cardiogenic inspiration and expiration with coupled spiracle leakage in resting blowflies, *Calliphora vicina*. *J. Exp. Biol.* **217**, 1543-1554.
Weis-Fogh, T. (1964). Diffusion in insect wing muscle, the most active tissue known. *J. Exp. Biol.* **41**, 229-256.
Weis-Fogh, T. (1967). Respiration and tracheal ventilation in locusts and other flying insects. *J. Exp. Biol.* **47**, 561-587.
Wisser, A. (1988). Wing beat of *Calliphora erythrocephala*: turning axis and gearbox of the wing base (Insecta, Diptera). *Zoomorphology* **107**, 359-369.