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RESEARCH ARTICLE

Structure of the thoracic spiracular valves and their contribution to unidirectional gas exchange in flying blowflies *Calliphora vicina*

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ABSTRACT

The operation of the thoracic spiracular valves was analysed using anatomical and physiological techniques. Dense spiracular filter trichomes impede a diffusive gas exchange. However, the hinged posterior filter flap of the metathoracic spiracle (Sp2) opens passively during upstroke of the wings and closes by the suction of the sub-atmospheric tracheal pressure during the downstroke, which supports a unidirectional respiratory airflow. The action of the interior spiracular valve lids was recorded by photocell sensors oriented above the enlarged spiracles and projected onto the screen of a video camera. The thoracic spiracles opened much quicker (approximately 0.1 s) than they closed (1 s), suggesting that the spiracular muscles are openers, as confirmed by experimental induction of muscle contraction. Simultaneous photocell measurement revealed that the first and second thoracic spiracles act concordantly. At rest, the spiracles were mostly closed or only slightly open (<1%). During intermittent short flights, the valves opened wide at the start of the flight for a short time, and in many cases opened again after the flight ended. Often, the opening was wider after the flight ended than during the flight itself. During long spontaneous continuous flight phases (up to 2 h), the valves were only slightly open (<5%), widening shortly after transient increases of wing stroke intensity. It is an amazing paradox that the spiracles were only slightly open when the requirement for O_2 was high during sustained flight. The advantage of generating sub-atmospheric pressure, supporting a unidirectional airflow with a P_{O_2} increase above the resting level, is discussed.

KEY WORDS: Auto-ventilation, Insect respiration, Tracheal pressure, Tracheae, Spiracles, Haemolymph, Opener muscle, Oxygen supply, CO_2 release

INTRODUCTION

Respiratory gas exchange in insects depends on a system of branched tracheal tubes with segmental openings called spiracles. The construction and proportion of the tracheae and spiracles are manifold, being adapted to the physiological requirements of the different insect orders and instars and even of different body segments. A general characteristic feature is the ability of the spiracles to open or close the tracheal apertures by valves for regulation of the inflow and outflow of respiratory air under the requirement of water retention.

The structure of the spiracles has been described for most insect orders, which show a great variety in their regulatory apparatus (Weber, 1933; Snodgrass, 1935; Nikam and Khole, 1989). The valves consist of various opener and closer components. Both can be muscles or elastic antagonists of cuticle or connective tissue. Whether the muscles work as openers or closers was generally deduced from their arrangement in the valve structure and seemed plausible without experimental evidence. There are visual observations in diapausing silkmoth pupae, locusts and cockroaches, but only few original recordings of the cyclic opening or closing behaviour of the valves (Schneiderman, 1960; Miller, 1973). In several publications, the opening behaviour was concluded from the measured CO_2 and H_2O release and O_2 uptake in different resting insects (Kestler, 1985; Lighton, 1988, 1996; Byrne and Duncan, 2003; Jögar et al., 2011; Wasserthal, 2014) or from other respiratory effects like abdominal movements with concurrent tracheal pressure changes (Schneiderman and Schechter, 1966). Anemometric effects were recorded at moth spiracles with thermistors (Wasserthal, 1981; Sláma, 1988). The O_2 increase was measured through specific pupal spiracles (Hetz et al., 1993). Because of technical difficulties, the valve action was measured only a few times during flight. A light reflection recording technique was applied at the mesothoracic spiracle (Sp1) of *Drosophila mimica* (Lehmann, 2001). Lehmann (2001) also 'calculated the spiracle opening determined with a geometric model for tracheal diffusion, assuming that spiracles are held continuously open during flight'. He concluded that the spiracle opening matches the metabolic need by diffusion. In flying *Caliphora vicina* at Sp1 and the metathoracic spiracle (Sp2), the tracheal pressure, CO_2 and H_2O emission were recorded (Wasserthal, 2015); during flight, a unidirectional respiratory gas flow through the anterior body was measured with an inflow of fresh air through the anterior spiracles (Sp1) and an outflow of CO_2-loaded air through the posterior spiracles (Sp2+Sp abdomen). The directed airflow is dependent on volume changes of the tracheal system due to deformations of the flight apparatus. This auto-ventilation leads to a sub-atmospheric pressure in the thoracic tracheal system with a mean sub-atmospheric pressure at Sp1 and a mean over-atmospheric pressure at Sp2, resulting in an increase of the tracheal P_{O_2} (Wasserthal, 2015). It was hypothesised that the pressure difference at the spiracles can only be attained by the involvement of some valve (Wasserthal, 2015). The aim of the present paper was to analyse the structural preconditions and activity of the valves at the thoracic spiracles during rest and locomotion, especially during flight, to test the hypothesis that valves contribute to unidirectional airflow as postulated previously (Wasserthal, 2015).

Two types of spiracle valves are known from Calliphorid flies: active and passive. Active valves are hidden behind spiracular filter structures. These inner spiracular valves can only be observed after removal of at least part of the filter trichomes. Uniquely in *C. vicina*, the posterior filter plate of the metathoracic spiracle is hinged and functions as a passive valve flap. It is an obvious candidate for influencing the tracheal airflow. Here, the movements of the flap or

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the valve lids were recorded together with the wing beat or leg movements using photocell sensors arranged behind the screen of a video camera.

It is generally accepted that the valve muscle in fly spiracles works to close the valve (Krancher, 1881; Hassan, 1944; Faucheux, 1973; Nikam and Khole, 1989). In contrast, preliminary observations indicated that the thoracic valve muscles in flies are openers. This was experimentally tested here using electrical, visual or tactile stimulations. Moreover, the structure of the valves was analysed by serial sectioning and 3D reconstruction to determine whether the muscle arrangement within the spiracular apparatus is more suited to functioning as an opener or as a closer.

MATERIALS AND METHODS

Animals

Blowflies (*Calliphora vicina*, Robineau-Desvoidy 1830) were captured from the field. They were examined immediately, or the F1 offspring of one vital female was bred and the adults were used after the fourth day at the earliest point after eclosion. The flies were kept alive in a flight cage from September 2015 to March 2016, hibernating in a cool winter garden. Data from specimens between the ages of 4 and 42 days were considered. There was no obvious fundamental difference in the behaviour or physiological reactions in flies of different ages. However, the older flies were much more likely to fly longer, for continuous periods of up to 2 h. The use of the wild *C. vicina* and F1 generation guaranteed powerful fliers. The procedure of using wild specimens and only the F1 generation avoided inbreeding and was a consequence of previous experience where flies from commercial or homogeneous laboratory stocks were short lived and incapable of flying persistently. The mean±s.d. mass of the flies was 82±14 mg (N=12 flies), and fluctuated by 24% during the experimental runs, depending on food intake. Several times a day, the flies were given a droplet of honey–water solution on a Styrofoam running ball. They were anaesthetised with CO_2 gas only for fixing and removal of the filter trichomes. The flies flew spontaneously without optical stimulation. After the experiments, the flies were still capable of flying and were released into the wild.

Recording of valve opening and closing

For all experiments, the flies were tethered (using Pattex, Henkel, Düsseldorf, Germany; Fixogum Marabu, Tamm, Germany) as described previously (Wasserthal, 2015). The action of the interior valve lids was observed after carefully removing the filter trichomes. Bleeding was avoided by using sharpened forceps and an ophthalmological scalpel. After ablation of the trichomes, the valve lids were observed and filmed using a Nikon D 800 camera combined with a Leica Macroscope M420 (Leitz, Wetzlar, Germany), 70× magnification, video-mode 60 frames s^{-1}. One or two silicon photocells of 3×3 mm were adjusted over the enlarged valve detail projected on the video screen at 35–70× magnification. When the fibre optic illuminated the closed valve lids, the reflected signal of their bright surface was high. When the valve lids opened, the dark holes of the tracheae were exposed and the light intensity was low. The bright–dark surface associations due to positional changes of the valve lids were converted into a voltage signal using a custom-made electronic set-up. The corresponding area of the dark (=open) surface was drawn and related to the maximum possible opening surface, corresponding to the inner contour of the peritrema frame, using a custom-made program for calibration. The percentage of maximal possible opening was correlated with the voltage values. In the figures, greater values on the ordinate correspond to more-open values (which measured darker); the detail measured by the photosensor is indicated in the inset icons with a red square. The video sequences with concurrent tracks using two Si photocells on two separate video screens were completed later by a third trace with a changed position of the photocell. This third trace was recorded offline on the same saved and repeatedly played video. This allowed simultaneous comparison of the behaviour of the inner lids at the Sp2 and the two orifices of Sp1.

The movement of the outer Sp2 flap was also recorded with this technique. However, as the movements of the dark brown flap were partly parallel with the optical axis, and therefore the contrast of the positional changes was low and difficult to observe, a calibration was not undertaken. The position of the Sp2 flap in relation to the position of the wing beat cycle was visible when the frequency of the illuminating strobe flash (Drelloscope, Moenchengladbach, Germany) interfered with the frequency of the wing beat at 145± 9.4 Hz (Movie 1), or when photographed with single camera shots at specific wing positions. In addition, high-speed videos were recorded with a Keyence microscope VW 9000 (objective VH-Z20, 200× magnification, recording rate 500 Hz, playback rate reduced to 7 frames s^{-1}; Keyence, Neu-Isenburg, Germany) using NIH ImageJ software (Movie 2).

Recording of flight and running

The movements of the wings were concurrently recorded by the air pressure pulses of the wing beat utilising a pressure sensor 1–2 cm below the tethered fly (Sensym SCXL 004 DN, Sensor-techniques, Puchheim, Germany). This contact-free measurement allowed visualisation of the flight intensity according to the different airflow pulses of the changing amplitude and frequency of the wing beats. The response times of the pressure sensors and the Si photocells are equally short and below 1 ms. The delay between the air pressure pulse of the wing beat and the optical signal varied between 3 and 33 ms (mean 25±11.5 ms, n=30 tested delays). These low values are of little impact for the determination of the onset and end of the wing beat phases and their correlation to the valve positions. The running activity was displayed similarly to the valve movements and utilised the intensity changes in reflectance of an irradiated leg moving in front of a dark background.

Data acquisition and analyses

Spiracle opening and wing beat data were continuously recorded on an Apple Powermac using a custom-made amplifier and a Powerlab AD-Interface with Chart 5.54 software (CB Sciences, Milford, MA, USA). The sampling rate was 200 or 1000 Hz. Student's t-test was used to determine the significance of differences between mean duration of spiracular opening and closing using Excel software.

Anatomy

For histology, the spiracles and the surrounding region were dissected and fixed in 4% glutaraldehyde–paraformaldehyde with 4% sucrose within 2 h of an initial 20 min evacuation and post-fixed in 1% OsO_4 in 0.08 mol l^{-1} phosphate buffer, pH 7.2, at 4°C for 1.5 h. For details of fixation and embedding in Epon, see Wasserthal (1999). Series of semi-thin sections were cut on an Ultracut 3 (Reichert, Austria) with diamond knives, stained with Toluidine Blue and analysed and photographed using an Axiophot light microscope (Zeiss, Oberkochen, Germany).

The 3D model of Sp1 was reconstructed on the basis of 36 serial sections using Maya 3.0 (Alias Wavefront, Toronto, Canada).

For scanning electron micrographs, the thorax was shock-frozen in 2-methylbutane, transferred to acetone and substituted by liquid CO_2 using the critical point drying method. The dried specimens

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were gold-coated for 3 min under Argon plasma at 25 mA and 2 kV (Hummer, Nanofabrication Facility, Stanford, CA, USA). The specimens were examined in a field emission scanning microscope at 1 kV (Hitachi S 800, Tokyo, Japan).

Stimulation of the spiracular valve muscle

The valve muscles were stimulated by inserting a V2A steel microelectrode with a tip diameter of 20 µm into the lateral cuticle below the spiracles, following perforation with a steel pin. The reference electrode was implanted in the haemocoel of the mesonotum. It was difficult to target the small valve muscle from outside and to obtain a response upon electric stimulation (N=3 flies, current of 60 mV, 1.3–2 µA, 100–300 ms). The reaction of the flies to electrical stimuli was greatest when the initial position of the spiracle was about half-open. The low reaction rate might have been caused by nervous inhibition. It was easier to get a response when frightening the intact fly (e.g. by touching an abdominal bristle with a cat whisker or shading the eyes).

RESULTS

The mesothoracic and metathoracic spiracles (Sp1 and Sp2) of *C. vicina* are relatively large (width 300 and 1000 µm, respectively), in comparison with the pore-like abdominal ones. They are accessible to videography and surgical intervention. They are interconnected by longitudinal trunks and supply the head and thorax. The seven pairs of minute abdominal spiracles were not analysed. The anterior three pairs connect the pair of large abdominal air sacs with the ambient air. The abdominal air sacs have no direct connection to the posterior metathoracic longitudinal tracheae. The longitudinal tracheal trunk in the metathorax is converted into a meshwork (plexus) of small tracheae (Faucheux, 1973; L.T.W., unpublished results).

Spiracle anatomy and the role of the valve muscle

In order to understand the regulatory apparatus, the cuticular components and the arrangement of the muscle were analysed by micro videography and by transmission light microscopy of serial semi-thin sections.

The first thoracic spiracle (Sp1) in *C. vicina* is a prominent oval structure with a maximum aperture surface of 0.234 mm^2. The external view reveals a dense roof formed by orange–brown branching trichomes extending from the cuticular frame (peritrema) around the spiracular opening (Fig. 1A). This felt-like filter leaves no visible opening and is not movable. It protects the delicate valve lids (Fig. 1B) and screens the space (atrium) between the trichomes and the inner valve from the ambient air. A larger tracheal space (vestibulum) behind the valve lids gives rise to a dorsal and a ventral tracheal trunk separated by a septum (Fig. 1C,D). The dorsal trunk leads to the thoracic tracheae and air sacs. The ventral trunk leads to the cephalic tracheae and air sacs.

The valve lids are lateral folds of the most exterior tracheal wall. Interiorly, they are flooded by haemolymph (Fig. 2F,H). The leading edge of the lids is strengthened by cuticular rods, which are sclerotised derivatives of the trachea.

The cuticular basis of the rods is broadened and forms a strong articulating structure, which is connected and fixed at the wall of the atrium and peritrema (Fig. 2). The rod base is thus kept in a stable position. A slender cone-shaped muscle arises from the ventrolateral cavity of the thoracic integument below the spiracle. It diverges and splits below the rod base and inserts inside the paired basic rods (Fig. 2D). The basal Sp1 was reconstructed as a 3D model on the base of serial semi-thin sections showing the arrangement of the muscle (red) and its attachment in the rods (Fig. 3).

The second thoracic spiracle (Sp2) has a wider aperture (0.38 mm^2) than Sp1. Sp2 is protected by two dark-brown filter plates, consisting of felt-like filter trichomes similar to those at Sp1 (Fig. 4A,C). The larger anterior plate is fixed. The posterior plate is joined to the cuticular frame (the peritrema) by a hinge and functions as an external valve flap (see below). The rim of the valve lids has a porous surface, which tightly adheres to the opposite lid when they are in contact. The muscle of the Sp2 is attached at the two rod bases, similar to that of the Sp1. It is more compact than the Sp1 muscle (Fig. 4F–H) and the tendons insert on the outside of the two rods (Fig. 5B).

Sp2 valve flap is passively moved by inspiratory and expiratory airflow correlated with the wing beat cycle

When at rest, if the flap was only loosely ajar over the aperture, it was observed to be adducted at the very beginning of a flight phase by the rising sub-atmospheric tracheal pressure and the subsequent suction. In the following wing beat cycles, the flap is pushed outwards during each upstroke by the expiratory airflow and closes during the downstroke, enabling inspiration through Sp1. These wing beat-correlated movements of the flap could be visualised by freezing the wing positions and flap movements simultaneously

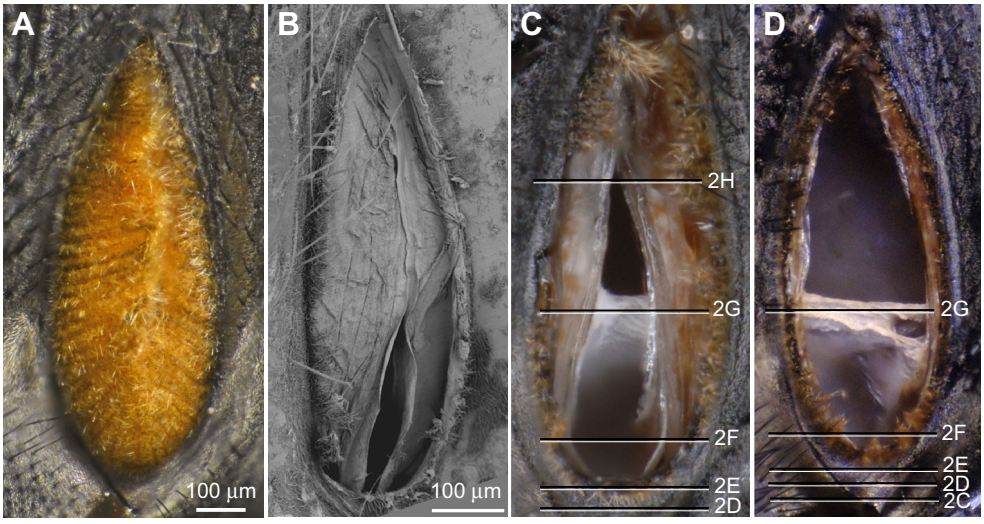

Fig. 1. Structure of the mesothoracic spiracle (Sp1). (A) Peritrema with felt-like filter trichomes (removed in B–D). (B) Scanning electron micrograph (SEM) after freeze fixation showing the valve membranes. Lids are open in the ventral part and tightly closed in the dorsal part. (C) Valve lids are retracted in a living fly, exposing the ventral and dorsal tracheal orifice. (D) Completely open valve lids of a CO_2-narcotised fly. Dorsal and ventral tracheal orifices and the septum between are exposed. The numbers in C and D (2C–2H) indicate the position of the plane of the histological sections in Fig. 2. Scale bar in B also applies to C and D.

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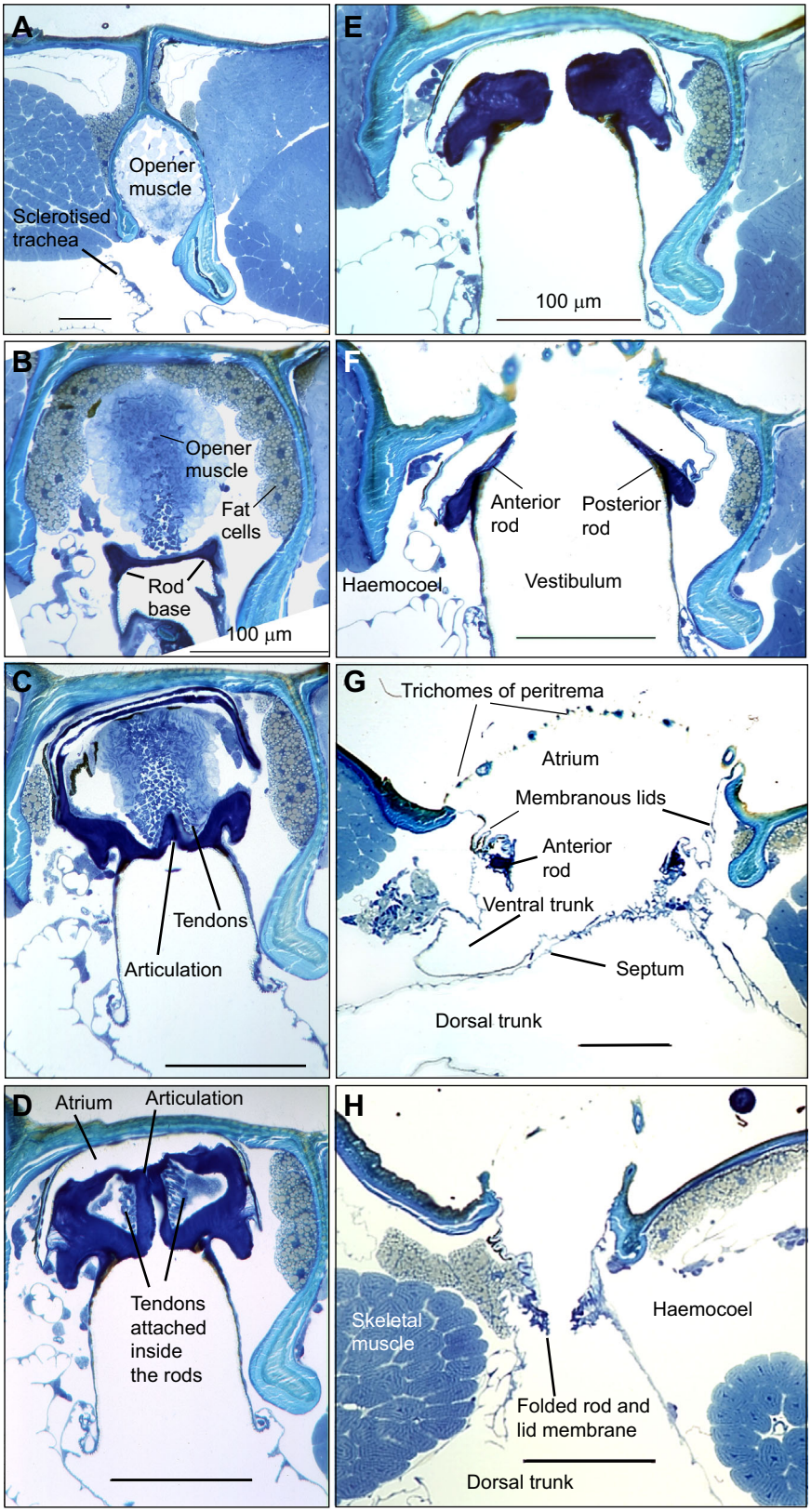

Fig. 2. Serial horizontal semi-thin sections through Sp1. Sections are from ventral (A) to dorsal (H). (A) Ventral-most cross-section of the opener muscle. (B) Sclerotised basal rod of spiracular lids. (C) Rod base fused with the external cuticle. Muscular tendons are arranged on both sides of the articulation of the rod base. (D) The enlarged basal parts of the rods are still interconnected. They contain a cavity where the muscular tendons attach. (E) The rods are separate and form the rims of the valve lids. Their connection with the outer cuticle is transformed into the membranous part of the valve lids. (F) Forceps-like part of the rods at the level of the ventral tracheal trunk. (G) Tapered rods at the level of the septum between the ventral and dorsal tracheal trunk. (H) Folded rods and the valve membrane at the level of the dorsal tracheal trunk. Scale bars: 100 µm.

with a strobe flash at a video frame frequency near the wing beat frequency of 145±9.4 Hz, or they were documented by serial camera shots (Fig. 6; Movie 1). To avoid frame interference, the movements were filmed using a high-speed camera-microscope with 500 frames s^{-1} (Movie 2). The movements of the wings and flap were also coordinated with the up and down cycles of the halteres and the abdomen opposite to the direction of the wing beat. The movements of the flap were not always visible, possibly because the flap was in an intermediate position or was only minimally deflected.

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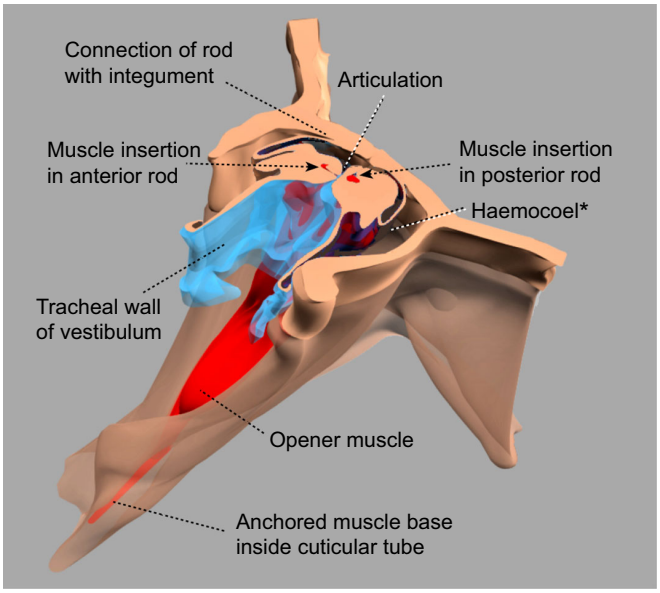

Fig. 3. 3D reconstruction of the basal valve region of the Sp1. The region shown corresponds to sections in Fig. 2A–D, displaying the opener muscle (red) with muscle insertions in the basal rods (arrows). Asterisk indicates the space of the haemocoel, which pulls apart the rod bases when it shrinks by dehydration in the dead fly. In both situations, the valves open.

Videographic recording of valve action

Sp1 opened continuously, progressing from ventral to dorsal, exposing first the ventral orifice, which leads to the cephalic trunk, and then the dorsal orifice, which leads to the thoracic trunk (Fig. 1B,C). The ventral orifice was always exposed from the very start of opening, with the consequence that the cephalic tracheae were supplied preferentially. The Sp2 valve lids opened and closed more equally like curtains. When open, they provided insight into the entire vestibulum, from where several thoracic tracheal stems arise.

Valve muscle morphology suggests their opener function

The muscles originate in a cuticular furrow ventrally below the spiracle and diverge towards the basal valve where they attach lateral to the articulation of the rod plate (Sp2) or inside the cavity of both basal rods (Sp1). Shortening of the contracting muscle is transmitted by tendons to the basal rods, which move apart like inverse forceps (Figs 4F–H and 5B–D).

Stimulation of the valve muscle induces lid opening

Opening of the lids was induced experimentally using electrophysiological, tactile or visual stimuli (Fig. 7). Even when the initial state of the valve was half-open, it opened wider after stimulation. Closing could never be stimulated. The opening process was significantly quicker than the closing process for both Sp1 and Sp2: Sp1 contraction 0.23±0.11 s, relaxation 0.95±0.59 s; Sp2 contraction 0.13±0.014 s, relaxation 1.02±0.21 s.

Differences in the mean duration of contraction and relaxation were statistically significant (Student's *t*-test, $P<0.00001$, $N=4$ flies, $n=18$ stimulations per fly). This shows that contraction is 4.1–7.8 times quicker than relaxation. The difference is even greater when the asymptotic end of the relaxation curve is considered (Fig. 7; Movies 3 and 4). The effect of the stimuli was recorded using the videographic and photocell technique. The muscles opened the valve lids maximally during fumigation with CO_2 for anaesthesia (Fig. 1D).

The spiracle opening behaviour during activity

At full rest, the respiratory gas exchange in *C. vicina* through the thoracic spiracles is facilitated by pressure changes induced by heartbeat reversals and a correlated leakage of the dorsal Sp2 (Wasserthal, 2014). As heartbeat reversals also continue with higher frequency during flight, this mechanism is also operative during flight (Wasserthal, 2015). The filter plates of the Sp2 leave a small gap above the leak, allowing for gas exchange through the leak, irrespective of the action of the valve muscle and the flap.

The young flies (tested 5 days to 1 week after eclosion) flew for only a few seconds or milliseconds, enough to change their resting place under natural conditions. During these short flight phases, the Sp1 and Sp2 valves opened wide immediately at the start of the flight, e.g. 17% in Sp1 dorsal orifice and 71.4% in Sp2 (Fig. 8A,B). The valves closed before the flight ended, but re-opened again after the flight ended, e.g. 8% in Sp1 and 20% in Sp2. In many sequences of short flight phases, the valves opened only to a minor extent during flight itself but opened more significantly directly after the flight ended (Fig. 8C). The intermittent flight phases with their increased spiracular valve openings seem to allow for CO_2 release, thus replacing the known macro-bursts of the discontinuous gas exchange. During continuous, long steady flight phases, Sp1 and Sp2 were unexpectedly only slightly open (<5%), but after intervals of 10–30 s, when the amplitude and frequency of the wing beat shortly increased, the valves opened wider with a delayed maximum peak after return to the less powerful wing beat (Fig. 9). During feeding and walking activity, the valves opened wide for longer than during flight (Fig. 10). The longest continuous open phases were recorded after the flies had sucked honey solution, which was then digested in instalments (Fig. S1).

Sp1 and Sp2 valve lids behave similarly

The measurements with two photocell sensors allowed parallel comparisons of the behaviour at Sp2 and the two orifices of Sp1 (Fig. 10). The opening behaviour of Sp1 and Sp2 was basically similar. The dorsal and ventral orifices of Sp1 opened in a rather concordant way, but the ventral orifice often opened first. Sp2 was open maximally for a longer period of time. The ventral orifice of Sp1 was more similar to Sp2, as it was exposed for longer than the dorsal orifice.

DISCUSSION

The external valve flap contributes to unidirectional airflow in the thoracic tracheae

The unidirectional respiratory airflow during flight, documented by the tracheal pressure difference between Sp1 and Sp2 and the directed CO_2 release through Sp2 and the abdominal tracheae (Wasserthal, 2015), is facilitated by the Sp2 valve flap. This flap is pressed passively open by the expiratory airflow during wing upstroke (Fig. 6; Movies 1 and 2). This coordinated opening with the wing beat has also been documented by a high-speed video filmed at 3200 frames s^{-1} in *Calliphora* (Jonathan Page, personal communication). During wing downstroke, the flap is adducted by the suction force of the sub-atmospheric pressure pulse, generated by the contraction of the dorso-longitudinal flight muscles, thus preventing inspiration through Sp2. This explains why almost all air is inspired though Sp1 during downstroke and expired through Sp2 during upstroke (Wasserthal, 2015).

The inner valve lids contribute to maintain a sub-atmospheric pressure and support gas exchange

In contrast to the passive valve flap, the opening or closing of the interior valve lids does not coincide with the single wing beat cycle,

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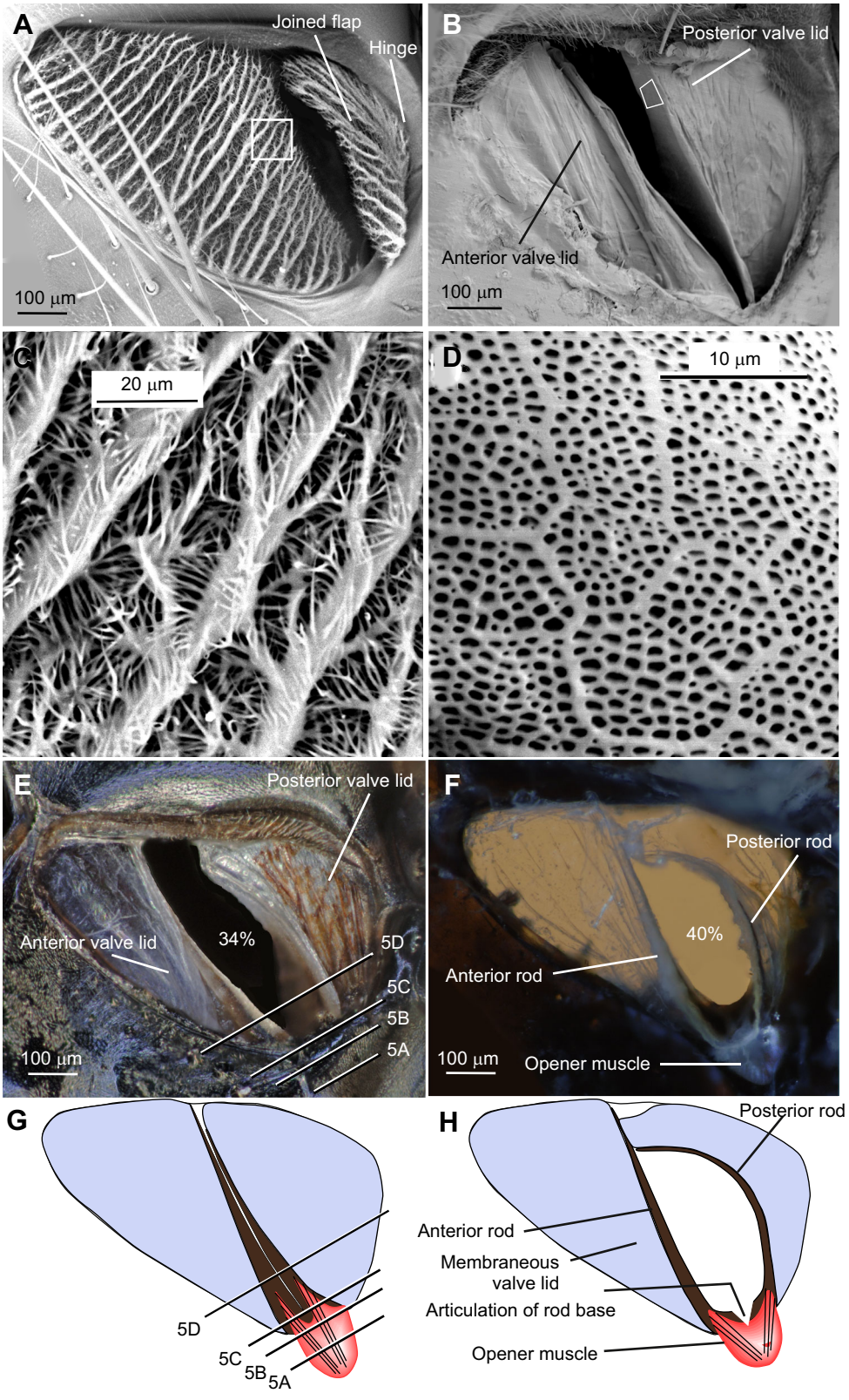

Fig. 4. Structure of the metathoracic spiracle (Sp2). (A) SEM of the outer filter plates. The posterior hinged flap is freeze-fixed in the open position. (B) SEM of the valve lids after removal of the filter plates. Lids open 13% of the maximum aperture. (C) Detail from A (boxed region) of the anterior filter plate with branching trichomes. (D) Detail from B (boxed region) of the porous surface of the rim of the posterior lid. (E) Sp2 of a living *Calliphora* with opened lids exposing 34% of the maximum aperture. (F) Interior view of Sp2 with valve lids and opener muscle; combined transmitted and dark-field illumination. Skeletal muscles and fat cells are removed. (G,H) Diagrammatic view of a closed valve (G) and an open valve (H). The numbers (5A–5D) in E and G indicate the position of the plane of the sections in Fig. 5.

but Sp1 and Sp2 valves during running and flying are open for longer periods and are basically concordant. As expected, these valves open wide at the start of short flight phases. Sometimes they open wide shortly before flight begins (Fig. S1). Generally they open wide again after flight stops (Fig. S2). A crucial question is why during steady flight the spiracular lids are typically only slightly open most of the time. It has been suggested that this allows the formation and maintenance of the documented sub-atmospheric tracheal pressure in the thorax, a precondition for the efficient inward convection of fresh air with the O_2 rise above resting level (Wasserthal, 2015). This flight muscle-generated respiratory airflow is distinguished from the conception of a pure diffusive gas

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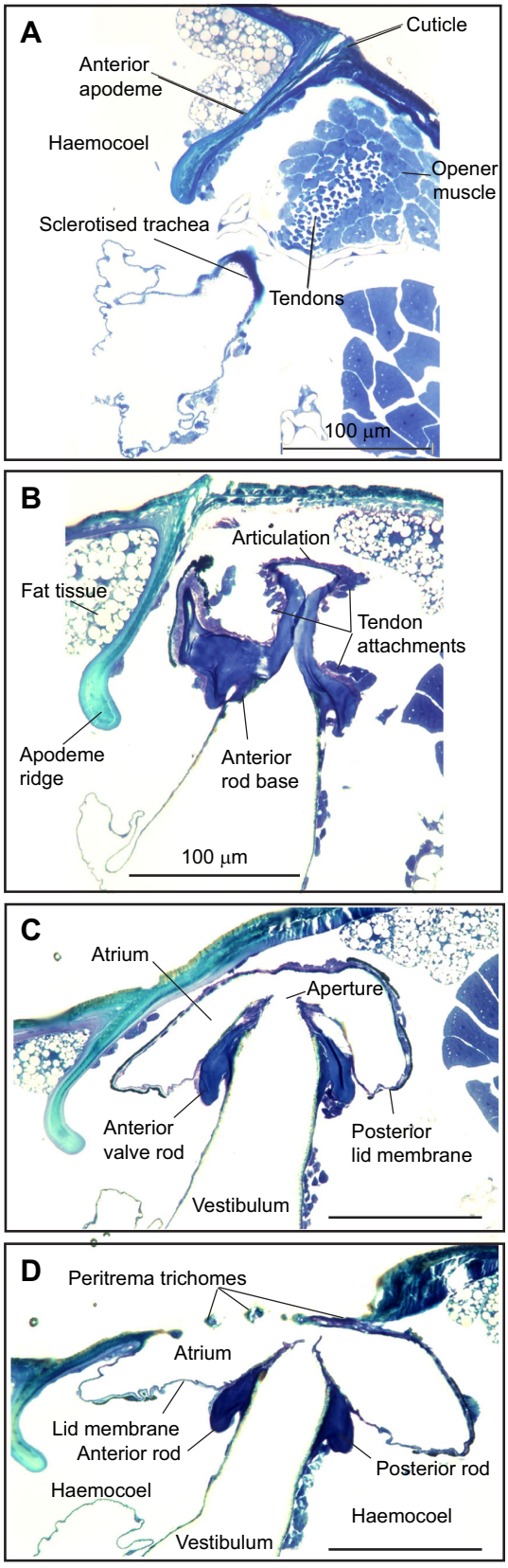

Fig. 5. Serial horizontal sections through Sp2 fixed in a slightly open position. (A) Ventral cross-section of the opener muscle and tendons in the centre. (B) The anterior and posterior rod bases are interconnected by an elastic articulation. The tendons of the opener muscle attach at the outside of the rods. (C) The forceps-like rods regulate the aperture between the atrium and the vestibulum. (D) Middle part of the valve with peritrema trichomes.

exchange during flight in small insects (Weis-Fogh, 1964; Lehmann, 2001). In addition, the O_2 concentration gradient between ambient air and the tracheal system increases by the consumption of oxygen and the transient retaining and buffering of CO_2 in the tissues and haemolymph. This is comparable to the volumetric O_2 consumption as a driving force for convective inflow into the tracheae, which was first described in lepidopteran pupae (Schneiderman and Schechter, 1966) and called 'suction ventilation' (Miller, 1974; Chown et al., 2006).

While the movements of the Sp2 flaps are clearly correlated with the wing beat cycle, allowing fresh air uptake through Sp1 and CO_2 release through Sp2, the influence of the inner valve opening on tracheal pressure and gas exchange exhibits a more complex effect. This is revealed by a comparison with published results (see figures in Wasserthal, 2015). Contrary to what was expected, during most of the longer steady flight phases, Sp1 and Sp2 were only slightly open (Fig. 9; Fig. S2). This is correlated with the mean sub-atmospheric pressure and the P_{O_2} increase in the scutellar air sacs when CO_2 and H_2O are released at the same time (see fig. 4A in Wasserthal, 2015). During intermittent short flights, a wide spiracle opening is documented by the P_{O_2} increase, attaining the maximum P_{O_2} value at or after the end of flight, congruent with valve opening (see figs 4B,C and 7 in Wasserthal, 2015). Corresponding to the opening pattern of the valves, CO_2 and H_2O are released not only during flight but also frequently before the start and after the end of flight (see fig. 6 in Wasserthal, 2015).

Spiracular valve muscles are openers

In contrast to the established opinion that the spiracular muscles in flies are closers (Krancher, 1881; Hassan, 1944; Case, 1956; Faucheux, 1973; Nikam and Khole, 1989), the thoracic spiracles in *C. vicina* were opened by the muscles and closed by elasticity of the cuticular apparatus and the tension of the haemolymph fluid behind the valve lids. This was shown by the opening reaction of the Sp1 and Sp2 valves to experimental stimulation. As the valve lids in resting *C. vicina* were closed or almost closed for most of the time, it is suggested that this is the relaxed condition. Further support that the muscles are openers comes from analysis of the time course of the opening and closing process. The spiracular valves opened 4.1–7.8 times quicker, and continuously, than they closed, especially when one considers the slow, asymptotic and often stepwise closing (Fig. 7; Movie 4). There is no experimental analysis or evidence for the closing function of the spiracular valve muscles in these flies. Their closing function has probably been deduced from the fact that in dead flies the valves stay open, thus being interpreted as the relaxed situation. This resulted in a drawing of Sp2 with a muscle converging towards the valve basis (Hassan, 1944, plate XX/3 and 4). Our preparations and serial sections of the muscles show, however, an inverse situation. The muscle fibres diverge from their ventral origin towards the attachments at the two sides of the articulated rod bases. In dead flies, the open state falsely resembles a relaxed condition. In dead flies, when the haemolymph volume shrinks by dehydration, the basal rods are pulled apart (Fig. 3, asterisk). The tension for closing is lost in the dead insects because it is dependent on the haemocoelic turgescence and adhesiveness at the inner side (on the rear) of the valve lids. It is assumed that the opening during CO_2 treatment of the fly is by contraction of the muscle mediated by CO_2-sensitive neurons. That spiracular muscles are controlled by neurons has been suggested by Case (1956) and can be postulated in those spiracles, which have muscular openers as antagonists to muscular closers (cockroaches, grasshoppers, bees,

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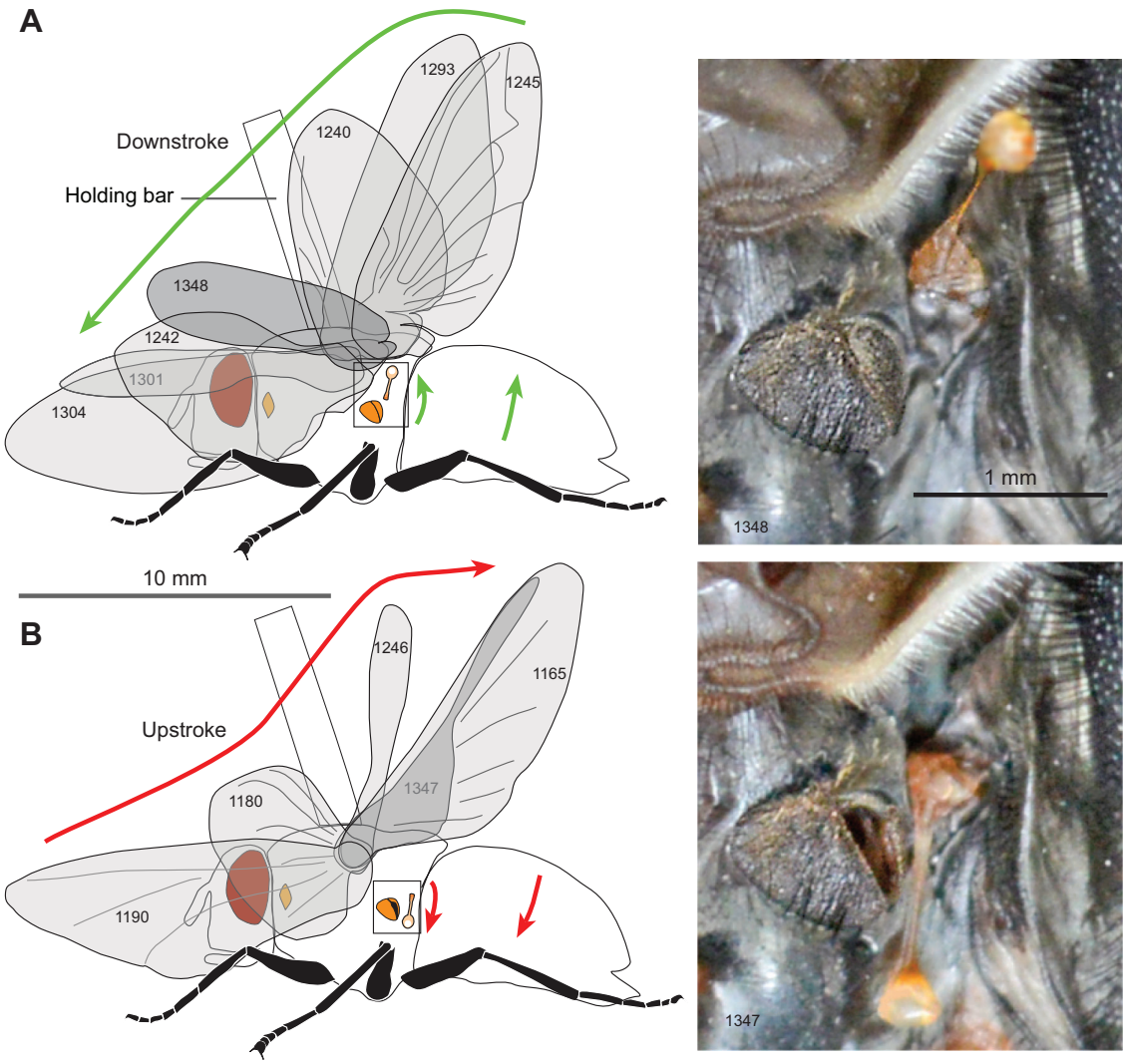

Fig. 6. Coordinated opening of the hinged Sp2 valve flap with the wing stroke cycle. Left, schematic diagrams of a lateral view of *Calliphora*, showing wing and abdomen positions and the positions of the haltere and valve flap in the boxed region. Right, video frame of the boxed region (scale bar applies to both images). (A) During downstroke of the wing (position 1348), the haltere and the abdomen move upwards and the valve flap closes. (B) During upstroke of the wing (position 1347), the haltere and abdomen move downwards and the valve flap opens.

ants and caterpillars, and pupae of papilionid butterflies with motoneurons and multipolar sensory neurons; Schmitz and Wasserthal, 1999). The spiracle closer muscle in pupae of *Hyalophora cecropia* has been shown to relax following exposure to high CO_2 (Beckel and Schneiderman, 1957). Both valve types react to high CO_2 by opening: the fly's valve opens by muscle contraction and the moth pupa's valve opens by muscle relaxation.

Convective airflow overcomes the resistance of the dense filter roofing of Sp1

It is hard to imagine that sufficient gas exchange by diffusion alone during flight is possible through the stiff and dense felt-like filter of the Sp1, even if the inner valves are fully open. The diffusive exchange of molecules in stagnant air should be very slow. The passive opening of the Sp2 flap during upstroke of the wings hints at a strong respiratory outflow confirming the unidirectional airflow. The respiratory gas flow, driven by the flight muscles, was documented by the rising O_2 level in the thoracic air sacs during tethered flight (Wasserthal, 2015). By not opening fully, the spiracular valves prevent an immediate equilibration with the atmosphere, thus maintaining sub-atmospheric pressure in the tracheae, which provides a convective inflow of fresh air at Sp1 against the resistance of the dense felt-like filter roofing.

CO_2 release via haemolymph at the tracheal plexus in the metathorax and air sacs near the posterior spiracles

While fresh air containing O_2 is streaming directionally through the ramified tracheoles into the O_2-consuming cells, the CO_2 is dissolved and buffered in the fluids of the tissues and haemolymph (Buck, 1958; Schneiderman, 1960; Miller, 1974; Kestler, 1985). The transition into the gaseous phase and flow back through the tracheoles against the incoming airflow is bypassed as the haemolymph with CO_2 comes in contact with tracheal surfaces like the tracheal plexus in the posterior metathorax (Faucheux, 1973) and the large abdominal air sacs in the anterior abdomen. Both tracheal areas are fully exposed to the thoracic haemolymph on its way past the lateral venous channels towards the abdominal heart chamber as in *Drosophila* (Wasserthal, 2007). In *C. vicina*, the heart chamber with the venous channels adjoins the anterior abdominal

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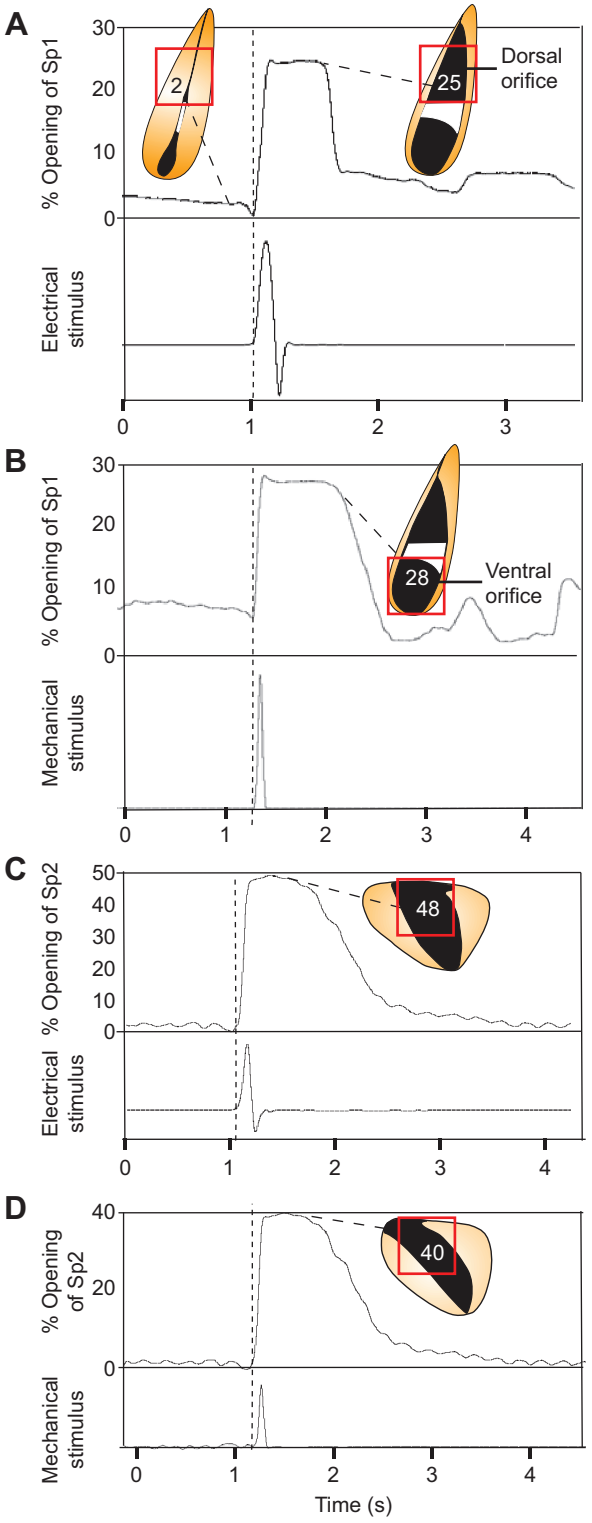

Fig. 7. Active opening of spiracular valves by different stimuli in *Calliphora vicina*. The reaction of the valve lids was recorded using light-sensitive photocells on video screens. Red squares indicate the sensor area. Upper traces show the course of the opening and closing process. Lower traces show the moment of stimulation. Sp1 and Sp2 icons represent characteristic situations. Numbers correspond to percentage opening. (A) Direct electrical stimulation of the Sp1 muscle, recorded at the dorsal orifice. (B) Tactile stimulation for induction of Sp1opening, recorded at the ventral orifice. (C) Electrical stimulation of the Sp2 muscle. (D) Induction of Sp2 opening by tactile stimulation. The opening process is much quicker than the closing process.

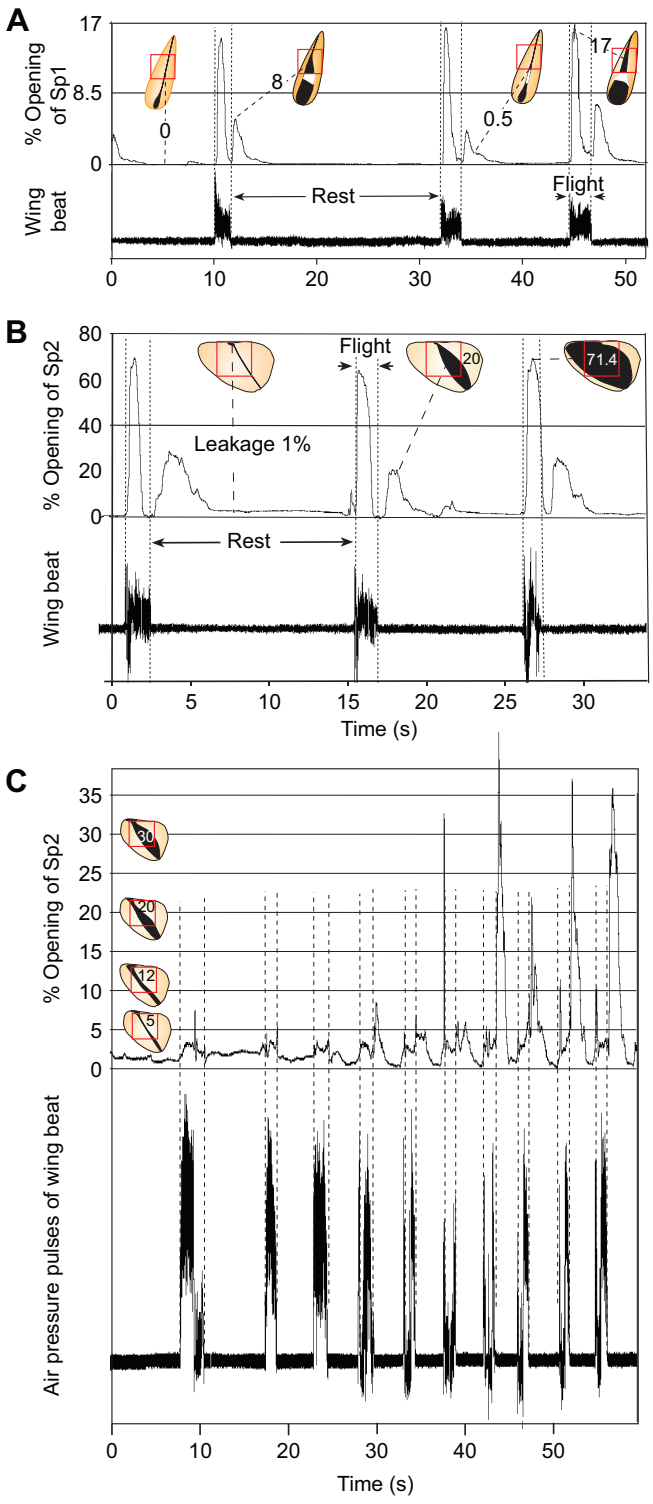

Fig. 8. Intermittent flight phases and concurrent opening of spiracles in *C. vicina*. (A) Sp1, dorsal orifice. (B) Sp2, aperture of vestibulum. Gravid female 9/2015 from the field, 96 mg. Upper traces show the video/photocell sensor signal. The valve opens during flight phases and shortly after each flight stop. Numbers beside and in icons correspond to the percentage of maximum possible spiracle aperture. Lower traces show the wing beat measured by pressure pulses of airflow. Sp1 and Sp2 icons represent characteristic situations. Red squares indicate the sensor area. (C) Intermittent flight phases with corresponding Sp2 openings increasing after the flight stops. During flight, only a slight opening occurs (<10%). A wider opening (≤30%) follows after each flight ends. Dotted lines indicate the start and end of the flight phases. Male 12/2015, 79 mg, 42 days after eclosion.

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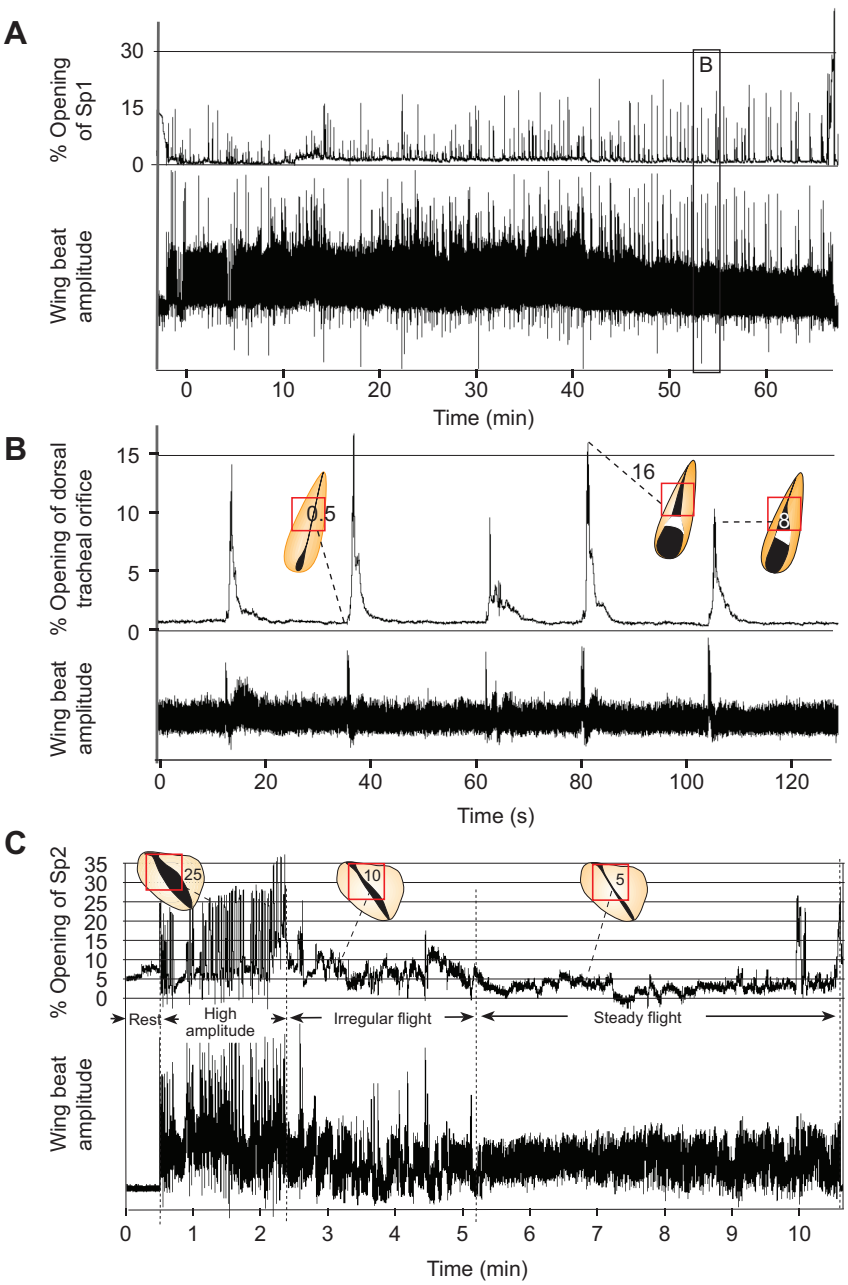

Fig. 9. Continuous steady flight in *C. vicina*. Upper traces show the video/photocell sensor record. Lower traces show the air pressure pulses of wing beats. (A) In a 1 h flight, the Sp1 valves are only slightly opened (<1%) but short wide openings occur following wing beats with enhanced amplitude. (B) Detail of A (boxed region). The opening is steeper than the closing. Female 10/2015, 30 days after eclosion, 92 mg. (C) A 10 min flight with an initial 2 min phase of intermittent high-amplitude wing strokes with corresponding wide Sp2 openings. During the second half of the flight phase, a stable flight with lower amplitude is performed while Sp2 opening is below 5%. Female 11/2015, 15 days after eclosion, 73 mg.

air sacs (L.T.W., unpublished). The accumulation of CO_2 in the abdominal air sacs during flight has been deduced from the reduced P_{O_2} measured in the abdominal air sacs during flight in *C. vicina* (see fig. 5 in Wasserthal, 2015).

Comparison of the opening behaviour of spiracle valves of *C. vicina* and *Drosophila*

The use of a similar technique for displaying the movements of the spiracular lids warrants a comparison of the results in *C. vicina* with those in *Drosophila* (Lehmann, 2001). However, the different morphological and experimental preconditions explain why no conformity between *C. vicina* and *Drosophila* could be obtained. The filter bristles of the filmed mesothoracic spiracles in *Drosophila* are too small to be removed to expose the valve lids to the micro videosystem. Preliminary tests showed that the reflection of the fibre optic illumination from the valve lids was disturbed by the superposed scattered light from the filter bristles, reducing the contrast of the different valve positions. The smaller size of *Drosophila* with a 100-fold lower mass (approximately 0.8 mg versus 80 mg in *C. vicina*) causes difficulties in displaying the action of the valve lids. In *C. vicina*, it was possible to selectively record the dorsal or ventral aperture of Sp1. Another difficulty in terms of recording the spiracle movements arises from the thoracic vibrations by the flight motor, which are transmitted to the spiracles as a whole. The need to enlarge the minute spiracles requires a higher magnification in *Drosophila* than in *Calliphora*, with the disadvantage of reduced resolution.

Drosophila does not have a movable flap at Sp2 nor a large pair of abdominal air sacs, in contrast to *C. vicina*. The absence of an external valve flap at Sp2 may indicate that in *Drosophila* the two thoracic spiracles work in a similar way and possibly do not contribute to a pressure difference between Sp1 and Sp2, which in *C. vicina* is a prerequisite for unidirectional tracheal airflow.

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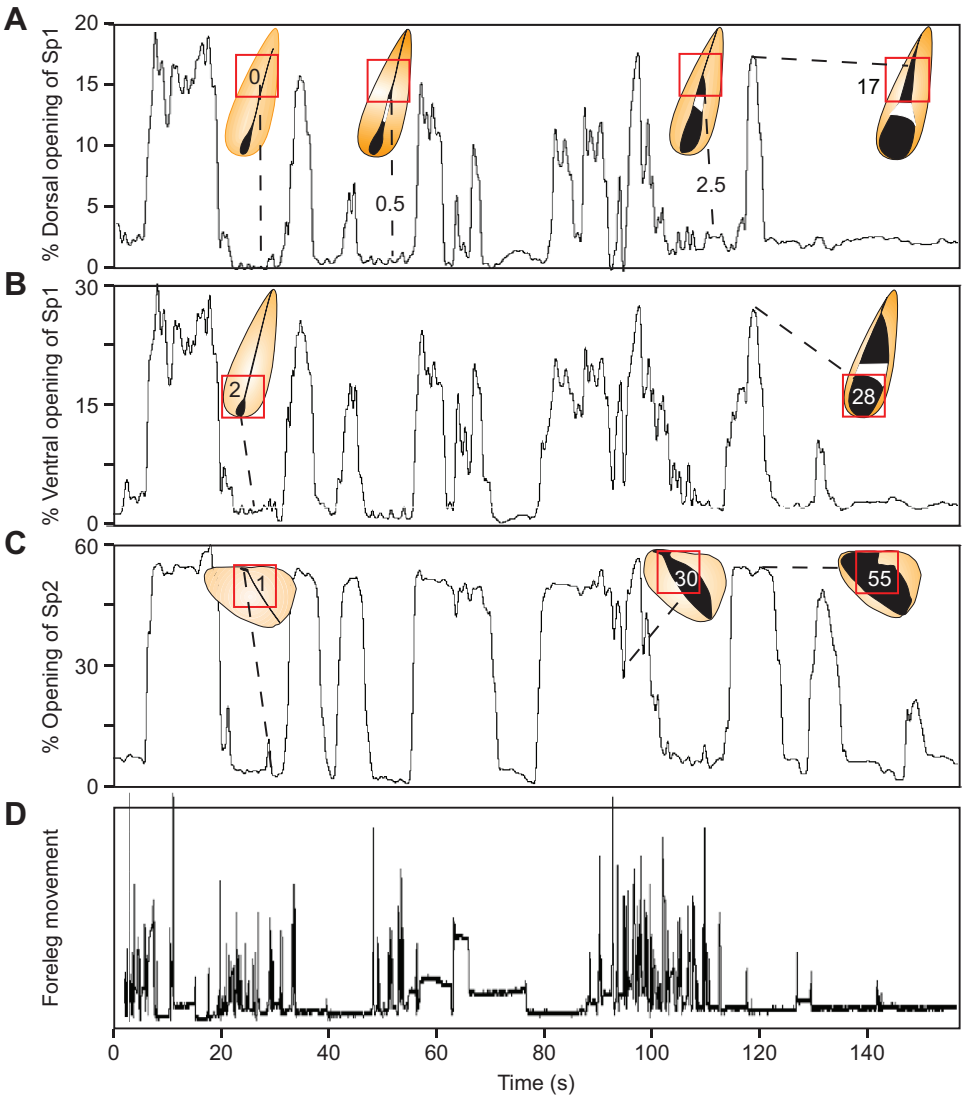

Fig. 10. Simultaneous recording of Sp1 and Sp2 behaviour during intermittent running activity. (A) Video/photocell sensor measuring the area in front of the dorsal orifice of Sp1. (B) Video/photocell sensor measuring the area in front of the surface of the ventral orifice of Sp1. (C) Video/photocell sensor measuring the area in front of the aperture of Sp2. (D) Photocell sensor recording of foreleg movements. The opening pattern shows a roughly concordant opening and closing of the two spiracles. The opening at the ventral orifice of Sp1 is more similar to the effects at Sp2 than that of the dorsal orifice of Sp1. Wide opening of the spiracles is not strictly concurrent with running activity; rather, it frequently parallels rest. Female 11/2012, 9 days old, 72 mg.

Under the premise that all spiracles are continuously held open and react in the same way during flight, in *Drosophila* the mean opening has been calculated to be modulated by between 50% and 100%, under the assumption that gas exchange is by diffusion alone (Lehmann, 2001). In *C. vicina*, Sp1 and Sp2 are only 5% open most of the time during steady flight, but they open wider (up to 20% in Sp1 and 35% in Sp2) in intervals of intermittent and slightly increased flight amplitude and after the flight ends.

Unidirectional respiratory airflow during flight – independent mechanisms developed in the neurogenic hawkmoth *Manduca* and the myogenic fly *Calliphora*

The unidirectional respiratory airflow of *Calliphora* is reminiscent of the flow-through mechanism in the hawkmoth *Manduca sexta*, which is similarly driven by the action of the flight apparatus (Wasserthal, 2001). Hawkmoths are also powerful flyers. In contrast to the myogenic *Calliphora*, which experiences only 1–2% shortening of the dorso-longitudinal muscles during downstroke (Boettiger, 1960), in hawkmoths with neurogenic flight muscles, the thorax shortens by 4.36–4.57% (George et al., 2012). This stronger shortening of the thoracic muscles in the moth leads to a protraction of the metathorax towards the mesothorax under closure of the inter-segmental cleft, screening Sp2 and preventing the inflow of air through Sp2 during wing downstroke. The screening and exposure of Sp2 corresponds to the role of the Sp2 flap in *Calliphora* as it supports expiration through Sp2 and inspiration through Sp1.

The flap reaction seems not to be the only mechanism to regulate the direction of flow. In some exceptional situations, the movements of the Sp2 flap were weak and scarcely visible. As unidirectional airflow has been shown to be directly coupled to the wing beat cycle, documented by the persistent pressure difference between Sp1 and Sp2 and the rise of tracheal P_{O_2} (Wasserthal, 2015), this suggests the occurrence of an additional valve-like component inside the tracheal system. This will be the subject of an x-ray computer tomographic analysis (L.T.W., unpublished results).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization, Project administration, Methodology, Visualization, Writing and Reviewing L.T.W.; Investigation: Physiology, Morphology, Videography: L.T.W.; Histology and 3-D Reconstruction: A.S.F.

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