RESEARCH ARTICLE

Periodic heartbeat reversals cause cardiogenic inspiration and expiration with coupled spiracle leakage in resting blowflies Calliphora vicina R.-D.

Lutz T. Wasserthal*
Dept. of Biology, University of Erlangen-Nuremberg, Staudtstr. 5,
91058 Erlangen, Germany

*Lutz.Thilo.Wasserthal@fau.de

Running title: Cardiogenic gas convection in blowflies

Supplementary material available online at:

Key words: insect respiration, circulation, tracheal pressure, tracheae, spiracles, oxygen, haemolymph, cyclic gas exchange, suction ventilation, CO₂-release, macro-burst, flutter phase, water retention.

SUMMARY
Respiration in insects is thought to be independent of the circulatory system because insects typically lack respiratory pigments and because oxygen transport occurs in the gaseous phase via a ramified tracheal system by diffusion and convection directly to the tissues. In the blowfly, as in other insects with periodic heartbeat reversal, the haemolymph is periodically shifted between the anterior body and abdomen, exerting alternating pressure changes on the compliant tracheae in the thorax and reciprocally in the abdomen. Simultaneous pressure and O₂-optode measurements show that, during negative pressure periods, the tracheal $P_{O_2}$ increases by 0.5 kPa. In the quiescent fly, tracheal $P_{O_2}$ is rather high (17.5–18.9 kPa), although the thoracic spiracles remain constricted. Microscopic video records and reflectance measurements revealed that the dorsal soft edges of the valve lips of the second spiracle leave a very small leak, which is passively widened during backward pulses of the heart. Thus, negative pressure, combined with increased leakage of the Sp2 valve enable inspiration in the thorax. The positive pressure periods are correlated with a new type of convective CO₂ micro-bursts as shown in flow-through measurements. The bulk of the CO₂ is, however, released after longer interbursts in macro-bursts with valves opening actively reminding of the open phase in a cyclic gas exchange (CGE). When the valves open, the $P_{O_2}$ in the thoracic air sacs unexpectedly drops by a mean of 2.75±1.09 kPa, suggesting a displacement of O₂ by the transient accumulation of CO₂ in the tracheal system before its release.

INTRODUCTION

Insects breathe by a branching air tube system called the tracheae, formed by lateral segmental invaginations of the exoskeleton. The blind tracheolar ends invade the tissues supplying them directly with oxygen. In many flight-adapted adult insects, the tracheae are partly enlarged to air sacs, which are suspended between the exoskeleton and the organs and occupy a vast part of the body cavity. The in- and outflow of respiratory air is regulated by valves behind the filter-protected atrium of the lateral openings, called the spiracles. The gas exchange in many resting insects is characterized by cyclic CO₂ release. These widely distributed cyclic mechanisms raised a prosperous research (Schneiderman, 1960; Lighton, 1996; Marais et al., 2005; Chown et al., 2006). The majority of recent investigations focussed on the impact of the physical ecofactors, such as temperature, humidity, and gas concentrations, on the diverse gas exchange patterns to evaluate their functional significance and ecological or evolutionary advantages.

The aim of this study is to analyse the assumed functional interplay of the periodic heartbeat reversals with the respiratory cycles and the involved structures such as the heart and spiracular valves. Their
action in quiescent Diptera has scarcely been visualized or recorded, because direct observation of most valves is difficult. They are hidden behind dense filter structures composed of ramified trichomes. Therefore the influence of the valves on gas exchange was deduced from the CO₂ output measured by flow-through experiments (Kestler, 1985; Lighton, 1988; Hetz et al., 1993), partly with cannulation to specified spiracles or compartments of the body (Wasserthal, 2001, Duncan and Byrne 2002), by thermographic-anemometric (Wasserthal, 1981; Slama, 1988) and volumetric-manometric measurements of expired or consumed air (Jögar et al., 2007; Karise et al., 2010; Jögar et al., 2011). A more immediate influence of the spiracles on the gas exchange was revealed by intra-tracheal pressure records (Brockway and Schneiderman, 1967; Kestler, 1985; Hetz et al., 1993) and oxygen uptake (Hetz et al., 1993; Wobschall and Hetz, 2004; Matthews and White, 2011). The gas exchange cycles are often characterised by a sequence of constriction, fluttering and opening of the spiracles (CFO) without or with coordinated bouts of ventilation movements (CFV) (Kestler, 1985) and are called discontinuous or in the absence of constriction cyclic gas exchange (DGC or CGE) (Lighton, 1996). It has been shown in \textit{Calliphora} and \textit{Drosophila}, that periodic heartbeat reversal causes changes in haemocoelic and tracheal pressure and volume alternating in the anterior body and reciprocally in the abdomen (Wasserthal et al., 2006; Wasserthal, 2007; Wasserthal, 2012) because the tracheal systems and haemocoels of both compartments are functionally separate (Faucheux, 1973; Wasserthal, 1999). Therefore, it was hypothesized that the haemolymph shift should ventilate the tracheal system by alternating between the anterior and posterior body. Under resting conditions, the tracheal pressure differs from the atmosphere because the spiracles impede immediate gas exchange and pressure equalization with the atmosphere by the more-or-less closed valves.

The action of the thoracic spiracular valves was analysed by pressure measurements directly at the intact spiracles. It was of interest to see whether the valves open and close synchronously or in a different manner. The simultaneous measurements at two spiracles was necessary for evaluation if the observation of one spiracle is representative for the other spiracles of the thorax. In order to avoid a blockage of the gas exchange by the tight sensor attachment directly at the spiracles, most pressure measurements and all oxygen measurements were performed by intubating one of the dorsal air sacs, which proved to be a mildly invasive procedure. For analysis of the influence of the spiracular valves on the uptake of oxygen and the release of the CO₂, simultaneous video records and reflectance measurements of the thoracic spiracular valves were accomplished. The influence of valve action on the emission of CO₂ and water loss was recorded by flow-through measurements concurrently with the air sac pressure registration. This study provides the first documentation of how tracheal pressure cycles based on periodic heartbeat reversals contribute to respiratory gas exchange.
RESULTS

Structure of the spiracles

C. vicina has two pairs of thoracic spiracles, the mesothoracic Sp1 and the metathoracic Sp2. Both spiracles can be closed by valves, which consist of opposing anterior and posterior membraneous lips (Fig. 1A-C). The lips are stretched and kept in tension by the underlying haemolymph. The free edges bordering the aperture are stabilized by sclerotized elastic bars, which are ventrally connected to the V-shaped muscle. In Sp2, the dorsal part of the valves is soft and flexible (Fig. 1C). The 7 pairs of abdominal spiracles are very small and have a simple circular opening with loosely arranged bristles in the atrium (Fig. 1A). Their inner valves were not visible under the experimental conditions. They were not analysed further. The surface of the thoracic spiracles is equipped with peritrema filter plates of ramified bristles, which protect the atrium and delicate valves behind. The filter bristles of the Sp1 form a dense stable roof (Fig. 2A). The Sp2 possesses an anterior fixed plate (Fig. 2G) and a posterior pin-jointed plate, which can be opened passively by a strong expiratory air stream (Fig. 2H). The filter plates were removed for observation of the valves. The thoracic valves are said to be closed by a muscle and to be opened by the elasticity of the sclerotized rims of the valve lips (Krancher, 1881; Hassan, 1944; Faucheux, 1973). There are, however, own observations and recordings, which suggest that these valve muscles are openers (Wasserthal & Fröhlich, unpublished).

Activity of the spiracular valves

During and immediately after CO₂-anaesthesia, the valves of Sp1 and Sp2 were widely open (Figs. 2F and N). Visual observations suggested that, after recovery in quiescent blowflies, the valves were mostly closed (Fig. 2B–D and 2I–K). The valves opened during and especially for a while after activity such as locomotion, grooming and feeding. When ceasing activity, the constricted valves seemed to be closed. After being fed, in the following hours the quiescent flies opened the valves at intervals (mean 21.6 ± 9.8 min (N=9, n=8 per fly) without visible exercise. The valves of Sp1 and Sp2 of both sides opened and closed generally synchronously, showing the same tracheal pressure in front of the spiracular openings (Fig. 3AB). However, the degree of the observed opening slit and correspondingly the pressure amplitude at the tubed spiracle could diverge (Fig. 3C). The opening slit of the valves varied continuously at values between 0% and below 8%. With widely opened valves (above 40%, Fig. 2F,N), the tracheal pressure was equilibrated with the atmosphere, and no pressure pulses could be recorded (Fig. 4). This happened especially after intensified activity.

Comparison of tracheal pressure at the spiracles and in the dorsal air sacs
The measurement of the tracheal pressure in front of the spiracles is problematic, because the closing of the valves interrupts the connection of the sensor with the tracheal lumen. Moreover, the partial opening of the valves can result in local pressure differences at the individual spiracle (Fig. 3C). The sensor blocks the connection of the spiracle opening with the ambient and may thus affect the pressure artificially of the entire thoracic system. To circumvent the possible disturbance of the spiracles, measurements of tracheal pressure were performed via intubations of the dorsal air sacs to obtain integrated values of the pressure and PO2 from the thoracic or abdominal tracheal system. Basically, the pressure curves show the same pattern no matter whether recorded at the spiracles or at the dorsal air sacs.

**Passive movements of the spiracular valves**

In addition to the active movements of the spiracular valves, a minute leak at the dorsal soft part of the rims of the valve lips of Sp2 could be detected (Fig. 5). This leak is difficult to see and was hitherto overlooked. During volume and pressure decrease in the thoracic haemocoel by backward heartbeat, the leak became passively widened (Fig. 5C–E). This explains why ambient air could be sucked into the thoracic air sacs and the PO2 increased even though the proper valve remained constricted (Fig. 5). The minimal extension of the leak during forward pulse period can be near 0% and expand to only 1% during backward beating (N=8, Fig. 5A->B). The widening of the leak can start from approximately 1% and extend to 5% (N=5, Fig. 5B->C) or from approximately 8.5% to 13.7% (N=3, Fig. 5D->E and supplementary material Movie1). The regular periodicity of the leakage was also measured by the changes of reflected light from the valve opening and the resulting exposition of the dark inner tracheal background (N=3, Fig. 6). In the video recordings, it could be shown that the valve lips vibrate in the frequency of the backward pulses, while they continuously increased the leak. The haemolymph on the rear of the valve lips is sucked in by the conical heart chamber connected via the lateral venous channels with the meta-thoracic spiracular region, as in *Drosophila* (Wasserthal, 2007; Wasserthal, unpublished).

**Oxygen uptake concurrent with heartbeat reversals**

Oxygen fluctuations in the scutellar air sacs were measured in intubated flies with simultaneous registration of intra-tracheal pressure or heartbeat. As postulated, the compensatory pressure decline in the air sacs of the anterior body by the negative pulses during backward beating was correlated with an oxygen increase in the anterior body (Figs. 7 and 8). At a Ta of 21°C, the PO2 ranged between a lower mean of 17.5±1.1 kPa and an upper mean of 18.9±1.1 kPa. (N=17, evaluated sequences: 1907 and time: 20h). At low metabolism in hibernating flies at a Ta between 3 and 19°C, intra-tracheal pressure cycles
continued with a coinciding O₂ increase during backward pulse periods leading to a mean PO₂ between 16.9±4.05 kPa and 17.9±3.2 kPa (N=7, n=523, Table 1 and supplementary material Table S1). The single lowest PO₂ of 4.5 kPa was measured at an intermediate Ta of 10°C. No general reduction of PO₂ in hibernating flies at low ambient temperature was recorded (supplementary material Table S1). The O₂ rise (ΔPO₂ per peak) during each negative pulse period ranged between 0.1 and 2.5 kPa with a mean of 0.5±0.2 kPa at 21°C. During sustained quiescence with leaking Sp2, the PO₂ remained constant at a high level (18.57±1.09, N=19), and no decrease could be seen towards the end of a several minutes-long period of resting heartbeat cycles. The mean PO₂ was also high when the O₂ peaks were weak and disappeared in the noise (mean 18.4±1.3 kPa, N=14, 20–30 sequences per fly). In a few cases, when the backward pulses were exceptionally omitted and pressure pulses remained positive for 4.8 ± 0.28 minutes (N=3, Fig. 8B), the mean PO₂ decreased by about 3±0.5 kPa in the thorax. After the reappearance of the rhythm with backward pulse periods and closed but leaking spiracles, the PO₂ returned to the original, higher values (Fig. 8B at 18:20).

Measurements in the abdominal air sacs showed the reciprocal correlation of O₂ increase during forward pulse periods with a rise in ΔPO₂ of 0.96±0.45 Pa (n=46 sequences), which produced a mean PO₂ level of 18.4±0.7 (N=3 females, evaluated sequences: 328 and time: 3.3 hours, Fig. 9).

**Convective CO₂ micro-bursts concurrent with heartbeat reversals**

The CO₂ output of the entire insect was measured by flow-through respirometry combined with recording of the intra-tracheal pressure of the scutellar air sac (N=14, evaluated sequences: 2287 and time: 13 hours). The CO₂ micro-bursts ranged between a minimum of 4.4 nmol s⁻¹ g⁻¹ and a maximum of 28.8 nmol s⁻¹ g⁻¹ (Table 2 and supplementary material Table S2). The pressure increase in the scutellar air sacs corresponding to periodic haemolymph accumulation in the thorax was in most cases correlated with a CO₂ micro-burst (Fig. 10A). The mean burst amplitude was 6.4±1.5 nmol s⁻¹ g⁻¹. While the O₂ rise in the anterior body occurred during backward pulse periods and in the abdomen during forward pulse periods, the moment of maximal CO₂ emission could change for some time within measurements in the same fly, possibly depending on phases of digesting crop contents (Fig. 10B). The coincidence of the CO₂ maxima with one of the pulse directions was not as reliable as the O₂ increase in the thorax and abdomen. As the CO₂ recordings by flow-through respirometry comprise the CO₂ emission of the entire body, it can only be deduced from the pressure conditions whether the emission came from the anterior or from the posterior body or from both parts. In 3 of 14 flies, distinct CO₂ bursts occurred during forward pulse periods and backward pulse periods (supplementary material Table S2, Fig. 10C). In these cases, the one burst during forward pulse periods was attributed to expiration of the anterior body and
the other one during backward pulse periods to expiration of the abdomen. In some sequences, both
bursts were equally strong and fused. Although being generally cyclic, the CO₂ emission never
decreased to 0.

**CO₂-macro-burst and oxygen drop during full spiracle opening**

In addition to the cardiogenic CO₂ micro-bursts in the leaky phase, during active opening of all thoracic
spiracles, the residual CO₂ was released as a macro-burst with a high mean amplitude of 273.5±151.4
nmol s⁻¹ g⁻¹ (N=9, 7–8 per fly at 22°C) (Figs. 10D and 12). The inter-burst phase between the CO₂-
macro-bursts averaged out at 21.55±9.82 min. The amplitude of the macro-bursts was up to 683.8 nmol
s⁻¹ g⁻¹ in well-fed quiescent individuals. The resting periods were often interrupted by phases of running
on the Styrofoam ball, grooming or regurgitating and re-imbibing the crop contents, accompanied by
irregular CO₂ release sparing the cyclic macro-bursts. All flies exhibited long phases of intermittent
activity without the cyclic CO₂ macro-bursts. As an unexpected result, the PO₂ in the scutellar air sac
dropped by ΔPO₂ of 2.75±1.16 kPa (N=11) when the spiracles fully opened (Figs. 11 and 12). This is
the contrary to what one would have expected (see discussion).

**DISCUSSION**

**Heartbeat reversals cause oxygen inflow by negative pressure and leaking spiracles**
The simultaneous intra-tracheal PO₂ measurements and pressure measurements confirm the hypothesis
that the cardiogenic negative pressure periods cause active inspiration. Because the negative intra-
tracheal pressure arises in the anterior body during backward (retrograde) periods and in the abdomen
during forward (anterograde) pulse periods (Wasserthal, 2012), it is evident that the heartbeat reversal is
causal for the force of this mechanism. The periodic PO₂ increase cannot be explained by a mere
physical effect due to pressure changes. Under negative pressure in a closed system, the PO₂ should
decrease and not increase. Moreover, the PO₂ reduction during pressure decrease is so small that it does
not significantly counteract the observed PO₂ rise. The determined pressure-dependent physical PO₂
changes between 0.02 and 2 Pa have an effect of only 0.004 and 0.4%, respectively, with regard to the
higher mean ΔPO₂ rise of 0.5±0.2 kPa.

It is a remarkable result that the PO₂ peaks occur and the relatively high mean PO₂ (17.5–18.9) remains
constant, although the thoracic spiracles are constricted in the neuromuscular sense. However, a critical
inspection of the spiracular valves revealed a very small leak of the Sp2 widening in the course of the
backward pulse periods of the heart. It is this leakage, increased by the negative pressure, that allows the
respiratory inflow and rise of the tracheal PO₂. At the first spiracle, no comparable leak could be
detected. The possibility cannot, however, be fully excluded that the ventral slit of the Sp1 valve is
slightly opened below 1%. The periodic PO₂ rise during the negative pressure periods (= forward pulses)
in the abdomen is assumed to function in a similar way of spiracle leakage.

Comparison of the cardiogenic gas exchange with the ‘passive suction ventilation’ (PSV) in other insects
PSV is a result of the discontinuous gas exchange cycle (DGC) based on the constricted phase, flutter
phase and open phase (CFO) of the spiracles described in lepidopteran pupae (Schneiderman, 1960;
Levy and Schneiderman, 1958; Brockway and Schneiderman, 1967). During the C-phase, the PO₂
decreases to a low value of 3 to 7 kPa due to O₂ consumption, and the tracheal pressure becomes sub-
atmospheric. When in the following F-phase the spiracles open shortly, the tracheal pressure increases to
nearly atmospheric values and fresh air is sucked in, preventing the PO₂ from sinking below the above
rather-constant low value. This gas exchange by simultaneous diffusion and inward convection of O₂ is
based on a great difference in partial pressure and hydrostatic pressure (Miller, 1974). It retains CO₂ and
H₂O while allowing maximal O₂ uptake in a N₂ equilibrium of outward diffusion and inward convection
(Kestler, 1985).

In the cardiogenic gas exchange of C. vicina, air is also sucked in by the negative intra-tracheal pressure,
but it is not supported by a high diffusive gradient of PO₂ between the tracheal lumen and the
atmosphere as in the PSV of the pupae. The suction mechanism in C. vicina is based on the negative
pressure in the haemocoel, which expands the tracheal lumen. The inspiration by tracheal volume
increase enables the airflow to reach the terminal tracheoles of the blindly ending tracheae or air sacs.
By contrast in the classical PSV in lepidopteran pupae, the suction force arises by the higher molar
oxygen uptake than molar CO₂ release due to buffering of metabolic CO₂ in the tissues and
haemolymph and delayed transition into the tracheae in the O-phase (Levy and Schneiderman, 1958).
The resulting negative intra-tracheal pressure in the C-phase is partly responded to by the reduction of
the tracheal volume setting the compliant tracheae under tension and shortening the abdomen. The
opening of the spiracles in the F- and O-phases leads to a relaxation of the tracheae and abdomen to
volume and length increases, respectively, with the consequence of inspiration. Both suction
mechanisms avoid stagnant air and the dependence on diffusion alone.

The cardiogenic form of active suction ventilation is performed by negative pressure periods alternating
in the anterior body and abdomen and passively adapting spiracle leakage. This stereotyped rhythm
fulfils a similar job as the flutter phases of the CFO-type in other insects, by enabling a longer lasting
convective and diffusive O₂ uptake, whereas CO₂ and H₂O have to diffuse against the inflowing air. In
the flutter phases, an active role of the spiracles in regulation of the inspiratory airflow is assumed, and the term “flutter” describes the sudden neuromuscular movements of the spiracular valves (Miller, 1981). By contrast, the proper valve mechanism in the leaky phase in *C. vicina* is not fluttering but remains constricted, and the Sp2 leak widens gradually and vibrates with heart pulses in the course of each backward pulse period of the heart and narrows in the course of the forward pulse period. A similar spiracular behaviour can be deduced in the abdomen from the O₂ peak during the negative pressure period in the abdominal air sac. A fluctuating leakage of the abdominal spiracles could not be detected here because of their microscopic dimension.

**Cardiogenic CO₂ micro-bursts and CO₂ macro-bursts**

A difference between the CFO-type and the cardiogenic gas exchange is the convective release of CO₂ in that compartment, which, due to the positive pressure periods, receives an increasing haemolymph volume, which becomes compensated by the decreasing tracheal volume and leads to a local and temporal separation of the interburst O₂ uptake and CO₂ micro-burst in the anterior body (Fig. 10A) and in the abdomen (Fig. 10B) or alternating in both (Fig. 10C). Independent from the cardiogenic gas exchange in *C. vicina*, macro-bursts of CO₂ emissions occur in longer intervals. They are reminiscent of the cyclic gas exchange (flutter-burst-type) in other insects with a long inter-burst phase and a short macro-burst phase. The inter-burst phase corresponds to the leaky phase in *C. vicina* with a variable number of heartbeat sequences. A correlation between inter-burst duration and number of heartbeat sequences, corresponding to the cardiogenic micro-bursts, could not be found. With a sequence duration of 27.5–39 s (data from Tables 1 and 2) between 32 and 46 heartbeat sequences could occur during one inter-burst phase with a mean duration of 21 min.

**PO₂ drop by accumulation of intratracheal CO₂ during spiracle opening**

The PO₂ drop in the scutellar air sac during the phases of full spiracle opening differs from published results in other insects with cyclic constriction, flutter and burst (CFB)-phases of the spiracles, such as lepidopteran pupae, beetles or cockroaches: The tracheal O₂ rises when the CO₂ is released as a macro-burst in the open phase (Punt et al., 1957; Levy and Schneiderman, 1966; Lighton, 1988; Hetz et al., 1993; Matthews and White, 2011). O₂ uptake occurs simultaneously with CO₂ release and small amounts additionally during the flutter phase. In *C. vicina* the O₂ uptake seems to be fully disconnected from the CO₂ macro-burst.

The probable reason for the PO₂ drop during open spiracles is the accumulation of CO₂ in the tracheae during the transition from the dissolved phase in the tissues and haemolymph into the gaseous phase just before and during release through the spiracles. Intra-tracheal CO₂ measurements are not available.
inside the flies, but it is known that the haemolymph pH becomes less acidic during the open phase in butterfly pupae (Hetz and Wasserthal, 1993) and in cockroaches (Matthews and White, 2011). The alkalosis in the haemolymph is an indication that the relative share of CO2 increases the tracheal $P_{CO2}$ and the total tracheal pressure and leads to an enhanced outflow of O2 into ambient air, which lowered the concentration of the O2. Measurements of O2 consumption in lepidopteran pupae showed a similar O2 drop ascribed to CO2 bursts (Jögar et al., 2011). The clear separation of the CO2 burst during open phase and the O2 uptake during leaky phase suggests that the active spiracle opening serves for CO2 release. This separation confirms results in _Hyalophora_ pupae (Levy and Schneiderman, 1958; Buck, 1962) and in the grasshopper _Taeniopoda eques_. In both insects the spiracle opening is triggered by a certain threshold of $P_{CO2}$ (Levy and Schneiderman, 1966; Harrison et al., 1995).

**Leaking gas exchange for water vapour retention**

The persistent, relatively high $P_{O2}$ of 17.5–18.9 kPa in the phases with constricted, but leaking spiracles in the quiescent _C. vicina_ is an argument against the hypothesis that the constriction phase prevents toxic damage by O2 radicals under low oxygen demand, which has been argued in connection with lepidopteran pupae (Hetz and Bradley, 2005). In adult flies, the maintenance of a high $P_{O2}$ with leaking spiracles favours the classical hypothesis of water retention, which has been suggested for saturniid moth pupae (Buck, 1962; Miller, 1974), and for Blattodea (Kestler, 1985; Schimpf et al., 2009), in which, under normoxic conditions, the $P_{O2}$ lies above 15 kPa (Matthews and White, 2011). Like in any active suction ventilation during inspiration over a short valve distance water vapour cannot diffuse outwards against the inflowing air stream (Kestler, 1985). But in _C. vicina_, water can be lost during positive pressure periods, which cause several CO2 micro-bursts during the interburst (Fig. 10A-C). The difference between the cardiogenic micro-bursts and the macro-bursts consists of a convective gas exchange versus a diffusive gas exchange of the macro-burst. As water molecules diffuse quicker than CO2 molecules, a diffusive gas exchange of CO2 should lead to 59.6 % higher water loss than a convective gas exchange of the same amount (Kestler, 1983; Kestler, 1985). This water retention can be deduced from the data of a similar example as shown in Fig. 12. A first comparison of the ratio of H2O evaporation per 1 nmol of CO2 emission during the interburst of 12 cardiogenic micro-bursts, which amounts to 0.33 nmol g$^{-1}$ with that of one macro-burst, which amounts to 0.79 nmol g$^{-1}$ H2O per 1 nmol g$^{-1}$ of CO2, results in a 58.2% higher diffusive water loss in the macro-burst. This preliminary evaluation confirms the above hypothesis that the cardiogenic mechanism is advantageous to withhold water as the number and duration of diffusive macro-bursts is reduced in favour of prolonged interbursts with convective CO2 micro-bursts.
Cardiogenic ventilatory mechanism in other insects

There are few known examples of the cyclic gas exchange in other flies, and no comparison exists with the periodic heartbeat reversals. In *Drosophila melanogaster*, CO₂ bursts occur in populations selected for desiccation resistance with a burst cycle frequency of 1.2 per minute (Williams et al., 1997). The CO₂ release during the ‘closed’ phase was discussed in regards to a possible leakage of the spiracles. In mosquitoes a similar burst cycle frequency of 1.1 per minute was recorded at a flow rate of 1000 ml min⁻¹ at 20°C (Gray and Bradley, 2006). In Tsetse flies, CO₂ macro-bursts have been recorded with a cycle frequency of 0.054 to 0.080 Hz = 3.2 to 4.8 cycles per minute (Terblanche and Chown, 2010). All these burst frequencies are high when compared with *C. vicina*. Fewer heartbeat sequences or only a single one might occur during one of these relatively short inter-burst phases. *Drosophila* with a heartbeat sequence length of 19–25 s (Dulcis and Levine, 2005; Wasserthal, 2007) and mosquitoes with a sequence length of approximately 20 s (Glenn et al., 2010) would perform 2 to 3 cardiogenic cycles per 1 inter-burst. In contrast to these fast-frequent macro-burst cycles, *Drosophila mimica* releases CO₂ bursts with pronounced flutter phases of the classical CFO-cycle alternating with long-lasting leaky phases of approximately 30 min. (Lehmann and Schützner, 2010). It is probable that these leaky phases are accompanied by numerous heartbeat reversals and reflect the cardiogenic ventilatory mechanism.

Periodic heartbeat reversal is a widespread phenomenon in insects (Gerould, 1929; Jones, 1977; Wasserthal, 1996), and it is predicted that it contributes to gas exchange in all cases in which the haemolymph is periodically shifted between the anterior body and abdomen. As distinguished from these flies, in adult giant silk moths, the CO₂-emission cycle is strongly coupled with the heartbeat sequence (Wasserthal, 1996) with periodic expansion and contraction of abdominal movements and superimposed bouts of peristaltic contractions at the end of each backward pulse period, which are coordinated with metachronic closing of the abdominal spiracles (Wasserthal, 1981).

Conclusions and Perspectives

Tracheal ventilation in quiescent *Calliphora* turned out to be mostly a side effect of periodic heartbeat reversal causing the changes in tracheal volume and appropriate spiracular valve leaks. Inspirations are shown to be only cardiogenic due to backward beating through increased leaking valves in the thorax and during forward heartbeat in the abdomen. The expirations follow mostly during forward heart pulses. Single abdominal pumping strokes coincide with the onset of each forward pulse period of the heart during the interburst (Wasserthal, 2012). They have no measurable effect upon the gas exchange whereas in other insects abdominal ventilatory movements are typical as volleys during the macro-bursts. In *C. vicina* the residual CO₂, which is retained in the tissues and haemolymph during the long
interburst, is released as a macro-burst during open phases. Thus, at rest, the respiratory gas exchange in the flies is water saving and energetically economic, because no special ventilation movements and spiracle muscle activity as in the flutter and open phase of other insects are required. This provides reserves for the high metabolic demands during flight, which in *Drosophila* is matched by continuous active opening of the spiracles (Lehmann, 2001). Whether in *C. vicina* during flight, periodic haemolymph shifts by heartbeat reversals continue and whether they contribute to the gas exchange will be addressed in a following study.

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

**MATERIALS AND METHODS**

**Animals**

Blowflies (*Calliphora vicina*, Robineau-Desvoidy, 1830) from the field and their offspring were used for experiments. They were treated and fed as described in a previously published paper (Wasserthal, 2012). The flies were anesthetized with CO₂ gas only for fixation and surgical treatment, but not during measurements. The mass of a well-fed adult was in the range of 53 and 120 mg (mean ± S.D. 84.5±16.4 mg). It diminished by 8–20mg when fasting within 24 hours at a temperature of 20–23°C and relative humidity of 60–82%.

**Recording the intra-tracheal pressure and heartbeat**

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

For measurement of the tracheal pressure inside the body, the dorsal cuticle with the underlying air sacs
was perforated and connected with a bi-tubated plastic cone using Pattex, which allowed the fly to be handled and clamped in the setup while being simultaneously connected to the pressure sensor (Sensym SCXL 004 DN, Sensortechniques, Puchheim, Germany) and to the O$_2$-optode (Fig. 1). The positive or negative pressure pulses at the spiracle and in the scutellar and the abdominal air sacs reflect the activity and direction of the heart pulses and are used as the reference for the periodic heartbeat reversal and the resulting haemolymph shift between thorax and abdomen. This method of pressure measurements has been described in detail and compared with electrophysiological records in a previously published paper (Wasserthal, 2012). The dead space of the sensor of 25µl and the 48 mm long tube connection to the spiracles or air sacs resulted in a 50% attenuation of the pressure signal. This was considered in the scaling of the curves. The response time was 7-10 ms and the time constant was 30 ms. In some flies the heartbeat was measured by extracellular electrical resistance myographs described previously (Wasserthal, 2012).

**Recording of intra-tracheal oxygen**

In addition to the dorsal pressure sensors, the flies were equipped with a fibre-optic optode (Microx TX3 AOT, PreSens, 93053 Regensburg, Germany). The tapered tip (diameter 50 µm) of this fibre was oriented directly above the perforation or inside the scutellar or abdominal air sacs, arranged beside the air pressure tube in the bi-tubated adapter cone (Fig. 1D). The measurements were run under controlled temperature, between 20 and 23°C, and with hibernating flies in an outside Faraday cage at ambient temperature between 2 and 19°C (Table 1 and supplementary material Table S1). The sampling rate of the optode was 1 Hz. Response time was 40 ms and 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 continuous use over several weeks without significant reduction in sensitivity and only slow, gradual loss in response time.

The influence of pressure changes on the PO$_2$ was checked by simulation experiments. In the first series, a micro-litre syringe instead of the fly was combined with the pressure sensor and the optode. Doubling the syringe volume (45 µl + dead space volume of 10 µl) by 50 µl at Ta 22°C in the closed system led to a PO$_2$ decrease from 19.8 to 9 kPa, a value that would also be expected theoretically. This shows that the measured increase of a PO$_2$ in the fly experiments during pressure decrease can only be explained by an inflow of ambient air into the open tracheal system.

In a second simulation series, the influence of pressure changes on the O$_2$-optode signal was tested in a closed 20-ml chamber. In the pressure range of 0.01 to 10 Pa, the corresponding physical PO$_2$ values were between 0.02 and 2 Pa, respectively. This was considered in the rise and drops of the O$_2$
measurements with the flies (See discussion).

Visualization and recording of spiracular valve action

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

Flow-through measurement of CO$_2$-emission and water loss

The CO$_2$ measurements were performed in a specimen chamber with controlled constant airflow (1000 ml/min) and adjustable pressure at controlled temperatures between 20 and 30°C. The volume of the specimen chamber was 20 ml and as small as possible (Fig. 1E) for recording at short response times for gas flow in the chamber. It was 1.4±0.2 s. The fly was glued at the mesoscutellum to a cannula, which at the same time served to fix the fly in position to the upper gum plug and connect the perforated air sac to the pressure sensor. A lateral port in the chamber allowed feeding and manipulation of a Styrofoam ball provided for foot contact in order to quiet the fly. The airflow stimulated prolonged running and grooming activities. Orientation of the head against the air stream appeased the flies noticeably. The chamber was connected directly to a CO$_2$/H$_2$O infrared gas analyser (LI-7000, LI-COR, Lincoln, NE, USA). Before entering the specimen chamber, the air passed a CO$_2$- and water-absorbing scrubber containing pellets of NaOH and the reference chamber. The pressure of the chamber was adjusted to a value between 10 and 50 Pa above ambient. The baseline of the CO$_2$/H$_2$O analyser and system was checked for drift after each experiment without a fly. The CO$_2$ output was calibrated between experimental runs in the absence of flies using a 50-ml syringe metering pump (Glenco Houston, Texas), simulating the release of a constant volume of pure CO$_2$ gas in steps at different flow rates (0.1–10 µl/s).
Data acquisition

Data were continuously recorded on an Apple Powermac or Powerbook using a custom-made amplifier and a Powerlab 8-channel AD-Interface with software (Chart 5.54; CB Sciences, Milford, MA 01757, USA). The sampling rate was 200 Hz.

ABBREVIATIONS

CFO Constricted – Flutter – Open
CFV Constricted – Flutter – Ventilation
CGE Cyclic Gas Exchange
DGC Discontinuous Gas Exchange Cycles
PSV Passive Suction Ventilation
Sp1 mesothoracic spiracle
Sp2 metathoracic spiracle

ACKNOWLEDGEMENTS

I wish to thank Thomas Messingschlager and Alfred Schmiedl for technical assistance and Prof. Manfred Frasch, Department of Developmental Biology, for laboratory use. I am also indebted to the reviewers for constructive comments and enhanced discussion.

FUNDING

This research profited from the generous support of the University Erlangen-Nuremberg.

REFERENCES


FIGURE LEGENDS

Fig. 1. (A) Semi-schematic median section of C. vicina showing the tracheal system with the projection of the lateral spiracles (orange) and position of the sensors on the punctured dorsum above the scutellar and abdominal air sacs. (B) First spiracle, (C) second spiracle of a living fly. The filter bristles of both spiracles are removed to expose the valve lips and the aperture (with % of the maximum possible opening). The rectangle corresponds to the projection of the sensor area of the Si-cell for recording of the opening and closing of valve lips. (D) Arrangement of pressure sensor tube and O₂-optode connected with the scutellar air sac. E) Flow-through chamber for measuring CO₂- and H₂O-emission and simultaneous tracheal pressure.

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

Fig. 3. Relative atrial pressure at ipsilateral or contralateral spiracles in resting male C. vicina (M15/00 at 22°C). The periodic pulse sequences reflect the heartbeat reversals. Black bars: Negative pressure pulse periods corresponding to backward heartbeat periods. (A) The
similar amplitude of the pressure pulses at left and right anterior spiracle indicates an almost identical opening of the valves at both sides. (B and C) Relative atrial pressure at left anterior and left posterior thoracic spiracle. (B) The similar amplitude of the pressure pulses indicates a rather identical opening of the valves at both segments as in A. (C) The amplitude of the pressure pulses may change inversely proportional between anterior and posterior thoracic spiracle when the valves behave differently. 0 = atmospheric pressure.

Fig. 4. **Intratracheal pressure changes in the scutellar air sac attributed to spiracle action in C. vicina (F13/09 at 22°C).** (A) A series of 31 heartbeat sequences interrupted by 3 phases with zero (=ambient) pressure: Black bars, negative pressure pulse periods = backward heartbeat. Tracheal pressure equalizes with atmospheric pressure when the spiracles are fully open. Pulses cannot be resolved. (B) Detail of A. Re-establishing of pressure pulses after 20 s with decreasing opening of spiracle 1 (insets according to frames from video records).

Fig. 5. **Leakage at the second spiracle varying with the periodic change of haemolymph volume in the thorax.** Filter plates removed. Left column: photographs on living flies (A–C) and video frames (D–E). Right column, schematic representation of analysed open areas. (A) Valve lips fully closed. (B) Leak very small (1.13%). (C) Leak at the dorsal soft edges of the lips increased to 5.26%. (D) Leak of 8.5% at the end of a forward pulse period, which at (E) increased at the end of a backward pulses period to 13.7%. See supplementary material Movie1.

Fig. 6. **Influence of the heartbeat reversals on the dorsal valve leak of spiracle 2 in C. vicina (F7/11 at 22°C).** Upper trace: reflection of valve lips on Si photocell. During widening of the leak the reflecting surface of the lips is reduced and the dark background behind the leak increases. Lower trace: Relative tracheal pressure pulses. Negative pressure pulses caused by backward heartbeat (black bars) positive pressure pulses caused by forward heartbeat (white bars).

Fig. 7. (A) $P_{O2}$ in the scutellar air sac (upper traces) and simultaneous myographic recording of heartbeat (lower trace) in male C. vicina (M15/09 at 22°C). During backward pulse periods (black bars), the $P_{O2}$ increases. (B and C) Simultaneous recording of intratracheal $P_{O2}$ and pressure in the scutellar air sac (F6/08 at 22°C). (B) Survey of 7 sequences.
(C) Detail of B. The PO2 increases during the negative pressure periods (backward pulse periods = black bars).

Fig. 8. Cyclic PO2 and pressure changes in the scutellar air sac during rest and different activities. The increase in PO2 during negative pressure pulse periods (black bars), due to the backward beating heart in female C. vicina (F33/09 at 14°C). (A) Regular periodicity of pressure- and PO2-changes. (B) Periodicity with different cycle lengths before and after a longer phase of positive pressure pulses and phases of running and grooming. In the long phase without negative pulse periods, the PO2 decreases without any visible activity. It increases during the following running and grooming activities and returns to a higher level after re-established heartbeat reversal. Black bars = backward pulse periods.

Fig. 9. Abdominal air sac pressure and PO2 in C. vicina (F31/09 at 23°C). (A) Survey of 13 sequences. (B) Detail of A. The PO2 increases during forward beating and decreases during backward beating (black bar) and during the pumping stroke (arrowhead) at the beginning of forward beating period. (C) Detail of A. Transition from forward pulses with negative peaks to backward pulses with positive peaks. The PO2 and air sac pressure are inversely proportional to conditions in the thorax.

Fig. 10. Flow-through respirometry measuring CO2 emission concurrent with intra-tracheal pressure of the scutellar air sac during the leaky phase (A-C) and during open phase (D). (A) Typical cyclic CO2 micro-bursts correlated mostly with the forward pulse period (white bars). During the backward pulse period (black bars), the CO2 emission decreases (female F9/09, 54 mg). (B) In a gravid well-fed female, the main CO2 peak partly coincides with the backward period, which shows a less negative pressure while a smaller CO2-peak occurs during forward pulse periods. (C) Some hours later, the CO2 emission peaks became equal during forward and backward pulse periods (F17/09 in B and C, 110 mg at 22°C). (D) CO2 macro-burst during spiracle opening and slight pressure increase above ambient without pressure pulses in the scutellar air sac (F9/09 at 22°C).

Fig. 11. Influence of neuromuscular opening of spiracle 1 on intra-tracheal pressure and PO2 in the scutellar air sac (F7/11 at 22°C). (A) Observed opening of Sp1 and the reflected light from the valve lips recorded by a Si-photodiode. When the valve opens, less light is reflected by the exposed dark tracheal hole. (B) Periodic intra-scutellar pressure pulses, which
are positive during forward heartbeat and negative during backward heartbeat. When the Sp1
fully opens, the pressure approaches to atmospheric pressure (= 0) and no periodic pulse
periods are visible. (C) \(PO_2\) drops down during spiracle opening and becomes restored when
Sp1 closes.

Fig. 12. **Emission of CO₂, H₂O and periodicity of PO₂ in resting *C. vicina* (F7/07 at 20°C)**
depending on heartbeat reversals and spiracle leakage and open phases. Upper trace
showing the periods with low amplitude cardiogenic CO₂-micro-bursts and high amplitude CO₂
macro-bursts during spiracle opening. Middle trace: H₂O peaks coincide with the CO₂ peaks.
Lower trace: Periodic oxygen peaks during backward pulse periods and \(PO_2\) drops during
spiracle opening in the scutellar air sac. Black bars, backward pulse periods.
Table 1. O$_2$ concentration and peak amplitude per cardiac sequence measured in the mesoscutellar air sac at different ambient temperatures ($T_a$).

<table>
<thead>
<tr>
<th></th>
<th>$N$</th>
<th>Heartbeat sequences</th>
<th>$PO_2$ Range (kPa)</th>
<th>$\Delta$ $PO_2$ /Peak amplitude (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Analysed n</td>
<td>Duration (s)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time (h)</td>
<td>No min$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>Mean $T_a$ 10°C</td>
<td>7</td>
<td>523</td>
<td>0.73 ± 0.5</td>
<td>82 ± 56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17h</td>
<td>±56</td>
<td>16.9 ± 4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Mean $T_a$ 21°C</td>
<td>17</td>
<td>1907</td>
<td>1.53 ± 0.61</td>
<td>39 ± 15.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20h</td>
<td>±15.6</td>
<td>17.5 ± 1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5 ± 0.2</td>
</tr>
</tbody>
</table>

Means are presented ±s.d., $N$, number of flies, $n$, number of heartbeat sequences.
Table 2. Cyclic CO₂ emission of entire flies (mean mass 84.5±16.4 mg) recorded by flow-through respirometry.

<table>
<thead>
<tr>
<th>Type of CO₂ emission</th>
<th>Ta (°C)</th>
<th>N</th>
<th>Heartbeat sequences</th>
<th>CO₂ Emission Range (min - max) (nmol s⁻¹ g⁻¹)</th>
<th>CO₂ Mean burst amplitude (nmol s⁻¹ g⁻¹)</th>
<th>CO₂ bursts during PPT</th>
<th>NPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiogenic Micro-burst Sp2 leaking</td>
<td>23</td>
<td>14</td>
<td>2287 13h</td>
<td>2.18 ±0.78</td>
<td>Sequence 27.5 ±9.8 s</td>
<td>4.4 – 28.8</td>
<td>6.4±1.5</td>
</tr>
<tr>
<td>Macro-burst Sp1+Sp2 open</td>
<td>22</td>
<td>9</td>
<td>69 24h</td>
<td>0.36 ±0.16</td>
<td>Interburst 21.5 ±9.82 min</td>
<td>34.2 – 683.8</td>
<td>273.5±151.4</td>
</tr>
</tbody>
</table>

Means are presented ±s.d.

N, number of flies; n, number of sequences; NPT, negative pressure pulse periods in the thorax; PPT, positive pressure pulse periods in the thorax; Ta, ambient temperature.