

RESEARCH ARTICLE

Influence of periodic heartbeat reversal and abdominal movements on hemocoelic and tracheal pressure in resting blowflies *Calliphora vicina*

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SUMMARY

In Calliphoridae and Drosophilidae, the dorsal vessel (heart and aorta with associated venous channels) is the only connection between the thorax and the abdomen. Hemolymph oscillates between the compartments by periodic heartbeat reversal, but both the mechanism and its influence on hemocoelic and tracheal pressure have remained unclear. The pumping direction of the heart regularly reverses, with a higher pulse rate during backward compared with forward pumping. A sequence of forward and backward pulse periods lasts approximately 34 s. Pulse rate, direction, velocity and the duration of heartbeat periods were determined by thermistor and electrophysiological measurements. For the first time, heartbeat-induced pressure changes were measured in the hemocoel and in the tracheal system of the thorax and the abdomen. The tracheal pressure changed from sub-atmospheric during backward heartbeat to supra-atmospheric during forward heartbeat in the thorax and inversely in the abdomen. The heartbeat reversals were coordinated with slow abdominal movements with a pumping stroke at the beginning of the forward pulse period. The pressure effect of the pumping stroke was visible only in the abdomen. Periodic hemolymph shift and abdominal movements resulted in pressure changes in the hemocoel and tracheal system alternating in the thorax and abdomen, suggesting an effect on respiratory gas exchange.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/215/2/362/DC1>

Key words: insect circulation, heart rate, abdomen movement, pressure fluctuation, tracheal system, hemolymph shift.

INTRODUCTION

Hemolymph transport in higher flies (*Drosophila* and *Calliphora*) is performed by the dorsal vessel, which periodically reverses its pulse direction. Hemolymph of the forward (anterograde) pulses enters the heart via five pairs of abdominal inflow ostia and leaves the dorsal vessel at the anterior aortal opening in the neck. Hemolymph returns to the abdomen via lateral venous channels, enters the anterior ostia of the conical heart chamber during backward (retrograde) pulses and leaves the heart through caudal openings (Wasserthal, 1982b; Angioy et al., 1999). A direct hemolymph exchange through the hemocoel is prevented by a pair of large air sacs in the anterior abdomen of *Calliphora erythrocephala* (Faucheux, 1973) and a septum in *Drosophila melanogaster* and *Drosophila hydei* (Wasserthal, 2007). These flies also lack a ventral hemolymph passage and a ventral diaphragm between the thorax and the abdomen, in contrast to many other insects (Miller, 1950; Richards, 1963). It is therefore expected that hemolymph is periodically shifted between the anterior body and the abdomen with the consequence of alternating pressure changes. In *Calliphora*, the tracheal system of the thorax and abdomen is also separated as the longitudinal tracheal trunks in the posterior thorax are modified to a narrow network in adults (Faucheux, 1973).

Heartbeat reversal in flies has been repeatedly described (Brazeau and Campan, 1970; Queinnec and Campan, 1975; Thon, 1980; Thon, 1982; Thon and Queinnec, 1976; Angioy and Pietra, 1995; Wasserthal, 1999; Dulcis and Levine, 2005; Slama and Farkás, 2005; Slama, 2010; Glenn et al., 2010), but it has rarely been convincingly recorded and analysed in intact flies; moreover, its functional implications remain

unclear. A controversy about the flow direction of high-frequency and low-frequency heart pulses has carried on until today. In a previous study on *D. melanogaster* and *D. hydei* I showed that the backward pulses have the higher frequency (Wasserthal, 2007) whereas Slama suggested that the backward (retrograde) pulses have the lower pulse rate (Slama, 2010). One aim of the present study was to analyse the pulse wave and direction using thermistor and electrophysiological recordings of the heartbeat. A correct attribution of pulse direction of periods with high-frequency and low-frequency pulses was also tested by measurements of hemocoelic and tracheal pressure. It was predicted that abdominal volume changes or ventilatory movements occur also in the blowfly and it has been assumed that abdominal activity may support heartbeats as in adult Lepidoptera (Wasserthal, 1976; Wasserthal, 1980; Wasserthal, 1981). Thus the main aim of this study was to determine whether the periodic hemolymph shift by heartbeat reversals produces periodic pressure changes with opposite effects in the thorax and the abdomen. The resulting periodic pressure changes in the tracheal system would support tracheal ventilation, as has been shown in Lepidoptera (Wasserthal, 1982a) and hypothesised in *Drosophila* (Wasserthal, 2007). As an application of sensors is difficult or impossible in *Drosophila*, the larger *Calliphora vicina* has been preferred as the experimental organism.

MATERIALS AND METHODS

Animals

Blowflies *Calliphora vicina* Robineau-Desvoidy 1830 were obtained and used directly from the field or their offspring larvae were reared on decomposing chicken meat or liver. After capture or eclosion,

the flies were kept in a tissue-covered cylindrical flight cage (45×60 cm, diameter×height). They could feed *ad libitum* on a mixture of honey, soft cheese and water. The reared flies were not used for experiments before the full development of flight muscles (Auber, 1969), at approximately 5 days to 1 month old. The measurements lasted several days. Only data from flies that survived the experiments in a vital condition were considered. They only exhibited clear resting rhythms after careful treatment during preparation, especially after the insertion of electrodes and dorsal punctures for pressure measurements. The tethered flies willingly seized a Styrofoam ball, which allowed them to run and to groom. During tethered flight they lost the ball, but could recapture it from a dish-like support below the fly. Between the experimental runs, the flies received water and food on the running ball. Most flies could be released after the experiments with the punctures sealed by a layer of Fixogum rubber cement (Marabu, Tamm, Germany). Before and after the experiments, the mass of the flies was determined. In both sexes the mass depended on feeding status. In females this was approximately 50 to 125 mg, depending also on the maturation stage of eggs. The males had a lower mass of approximately 45 to 90 mg. The mean (\pm s.d.) mass of F1 offspring was 66 ± 21 mg ($N=26$). Males were more difficult to equip with sensors because of their smaller size. Therefore, females were preferred in the experiments (supplementary material Tables S1–S3). Moreover, the largest flies were females from the field, which had entered the house (mean \pm s.d. mass= 87 ± 25 mg, $N=23$).

Recording of heartbeat by thermistors

Thermistors allow the measurement of pulses of the tubular heart below the intact body surface without fixing the insect in a stationary setup. The method utilises the effects of natural or artificial thermal gradients on unheated thermistors (T-method) (Wasserthal, 1980) or convective/conductive effects on slightly heated thermistors (C-method). For measurements of heat-marked heart pulses, Veco micro-thermistors ($2\text{ k}\Omega$ at 25°C , diameter 0.1 mm; Victory Engineering Corp., Springfield, NJ, USA) were attached with surgical tape to the cuticle of abdominal tergite 3 or 4 above the corresponding heart segments. With the thermistor at ambient temperature, the pulse direction was clearly recognised when the

hemolymph of the thorax was raised by a change in temperature (ΔT) of 1.5 to 2.5°C using a soldering bit, which at the same time served to fix the fly at the mesonotum. Backward (retrograde) pulses transported heat to the abdominal heart (Fig. 1A). As an alternative method, heat was applied by a laser beam (5 mW He-Ne Laser; www.conrad.de), heating the hemolymph by a ΔT of 1.5 to 2°C anteriorly, between or behind the measuring thermistors (Fig. 2). Alternatively, in the C-method, the thermistors were heated by a ΔT of 1.7 to 1.8°C supplying the Wheatstone bridge current with a higher voltage (1.5 V instead of 0.25 V as in the T-method). This allowed the visualisation of heart pulses and local hemolymph accumulation by their convective and conductive effects. As a disadvantage of the C-method, the single pulses tended to disappear in the steep changes in temperature. The pulses were, however, visualised using a band-pass filter, which suppressed events slower than 0.5 Hz and noise above 20 Hz. Using this procedure, the pulse rates were analysed on a broad data basis (Table 1, supplementary material Table S1). Data were evaluated from fully resting flies at a mean temperature of 21°C , without phases of grooming, feeding or running. The interpretation of the temperature effects on this C-method has been tested in physical simulation experiments (Wasserthal, 1980). This non-invasive method has been introduced in connection with records of moth hearts and pulsatile organs (Wasserthal, 1976) and has been reviewed (Miller, 1979). It has already been successfully applied in Lepidoptera, Coleoptera, Hymenoptera and Diptera (Wasserthal, 1982b; Wasserthal, 1996; Wasserthal, 1999; Hetz et al., 1999; Lubischer et al., 1999; Slama and Miller, 2001). This method allowed the thermistors to be mounted without anaesthesia and was preferred as a reference recording in combination with other techniques.

Extracellular electrical resistance measurements

As a more direct measurement of heartbeat, paired steel electrodes were placed on the left and right side of the anterior heart chamber–pericardial complex and at the fourth heart segment. The changes in electrical resistance, which were recorded, resulted from alterations in the distance between the heart muscle and the recording electrode and from changes in the electrical conductance across the dorsal vessel. Contraction of the heart (=systole) resulted

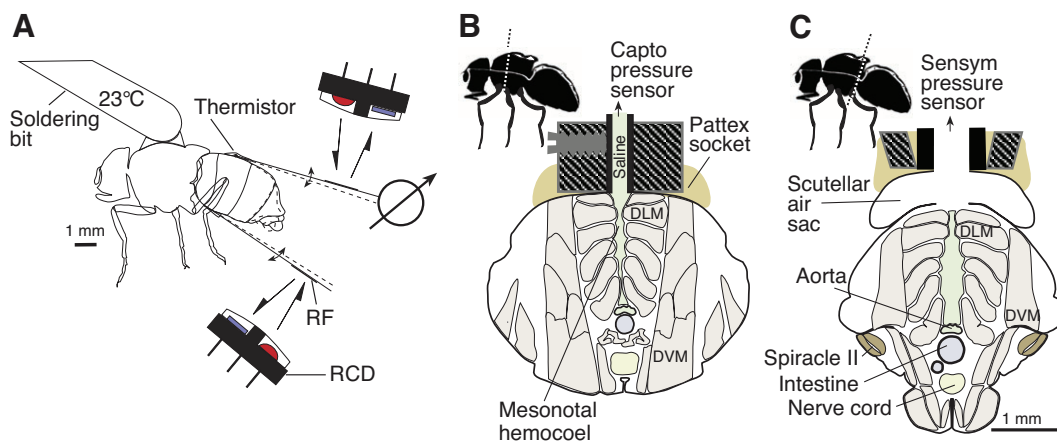


Fig. 1. (A) Setup for parallel recording of heartbeat and abdominal movements. A thermistor at the third tergite under heat marking of the thoracic hemolymph by a soldering bit records the pulse direction. Two infrared reflex coupler devices (RCDs) register the changes in distance to the microprismatic reflector foils (RFs) caused by abdominal movements. Sensors and reflectors are not to scale. (B) Cross-section of the anterior mesothorax with the position of the outlet to the hemolymph pressure sensor. (C) Cross-section of the posterior mesothorax with the outlet to the tracheal air pressure sensor. DLM, dorsal longitudinal muscle; DVM, dorso-ventral muscle.

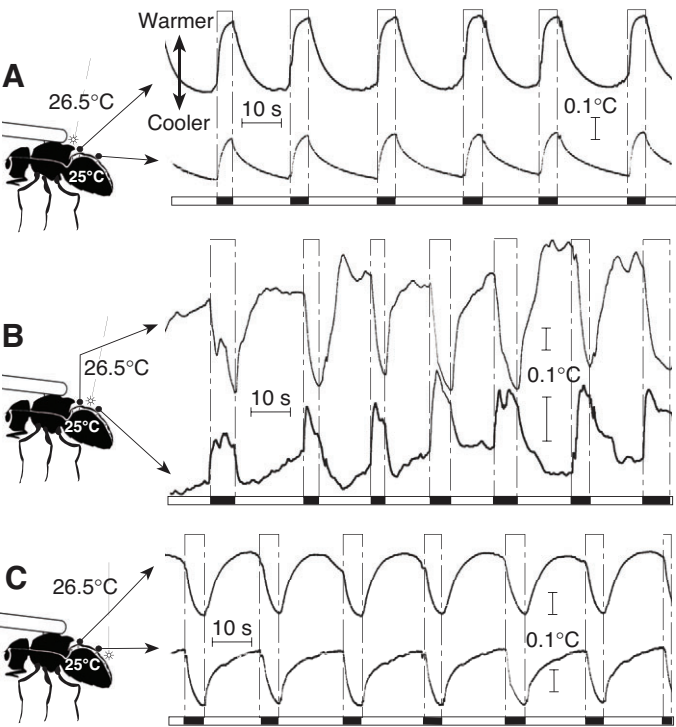


Fig. 2. Periodic heartbeat reversal in *Calliphora vicina* recorded with thermistors (T-method) above heart segments 3 and 4, and local heating by a laser beam. (A) Heating at the metathorax; shorter pulse periods at the third and fourth segments exhibit warming pulses. (B) Heating between the thermistors; temperatures of the anterior and posterior thermistor sites are inverse. (C) Heating at the fifth abdominal tergite; longer pulse periods exhibit warming pulses, and hence are forwards (white bars), whereas shorter ones are backwards (black bars).

in a negative peak. The V2A-steel electrodes were 20 μ m in diameter and their insertion through the inter-segmental fold directly beside the heart tube produced no lasting damage. Although the immediate contact of the recording electrode to the heart was essential, the reference electrode was implanted at a greater distance, usually 1 to 2 mm from the recording electrode. The electrical signal was low-pass-filtered by 20 Hz and amplified with a custom-made amplifier. This minimally invasive technique was also used to verify the data obtained by the more cautious thermistor method and was found to be superior for recording pulse velocity. This method has been introduced under the name ‘impedance conversion measurement’ and has been used successfully to record heart pulses in hawkmoths and bumble bees (Heinrich and Bartholomew, 1971; Heinrich, 1976) (for details, see Miller, 1979).

Recording of abdominal movements

Abdominal movements were video recorded from the lateral view (Canon EX1, Ohta-ku, Tokyo, Japan) at 25 frames s^{-1} , and then transformed into time-lapse movies with 18 \times acceleration using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (supplementary material Movie 1). In addition, positional changes of the third abdominal tergites and sternites were measured using position-sensitive infrared (IR) reflex coupling devices (RCDs; www.conrad.de) (Fig. 1A). The IR beam was reflected by a microprismatic reflection foil (3M Scotchlite 5870, St Paul, MN, USA). The reflection foil was not attached directly to the sclerites but 20 mm behind the abdomen on the thermistor wires and to another wire glued to the sternite. The RCDs were installed with micromanipulators opposite the reflex foils. The operating distance range between the RCD and the reflecting foil was between 2.5 and 3.5 mm. A step of 1 μ m corresponded to a 50 mV sensor output. However, because the movement of the tergites and sternites was not simply an up and down movement but rather an inclination, the angle with which the fine copper wire with the fixed reflection foil moved towards or away from the RCD was used to scale the ordinate (in degrees). By application of the foil on the thread, the lever effect offered the advantage of a mechanical amplification of sclerite movements. In some individuals, activity of the thoracic spiracular valves was observed with a binocular microscope after removal of the filter structures or recorded with a digital camera (Canon 60D).

Hemolymph pressure measurements

Hemolymph pressure was recorded in the dorsal hemocoel below the mesoscutum (Fig. 1B) and dorso-laterally below the fourth tergite of the abdomen. The notal cuticle was perforated in CO₂-narcotised flies and connected to a pressure transducer (Capto SP 844, 3193 Horten, Norway; sensitivity: $\Delta 1$ mV=26.5 Pa). A metal or plastic cylinder glued to the punctured cuticle allowed the insertion and adjustment of the tip of the syringe needle of the sensor setup in the insect. To facilitate abdominal movements, the syringe was connected to a flexible plastic tube. The pressure sensor was attached by a plastic dome to a 1 ml syringe containing saline (Ephrussi and Beadle, 1936). Saline was needed to transmit the hemolymph pressure to the sensor and to avoid air bubbles. An eventual excess of saline, which increased hemolymph pressure, was always reduced by the flies within the next 2 to 9 h by the observed excretion of fluid. The influence of the hemolymph volume increase on the pressure curves and heartbeat periodicity was tested by application of 10 or 20 μ l saline laterally on the abdomen. By puncturing the intersegmental membrane, the droplet was sucked in. The flies always restored the original negative hemocoelic pressure by diuresis, as normally occurs after eclosion and wing inflation (Cottrell, 1962). The hemolymph pressure data were calibrated using a mechanical barometer PMK04 A109 -2.5 to +1.5 kPa (PKP Prozess Messtechnik, Wiesbaden, Germany). The delay between

Table 1. Heartbeat frequency and duration of sequences, pulse periods and pauses in *Calliphora vicina*

	N	T _a (°C)	Sequences		Duration of pulse periods and pauses (s)				Pulse rate (Hz)	
			No./min	No. min ⁻¹	Forward	Pause	Backward	Pause	Forward	Backward
Thermistor recordings	12	21.0 \pm 0.8	442/231	2.3 \pm 0.97	22.9 \pm 18	0.9 \pm 0.82	7.4 \pm 4.39	0.97 \pm 0.65	3.4 \pm 0.45	4.9 \pm 0.57
Electrophysiological measurements	14	21.6 \pm 0.5	342/216	1.8 \pm 0.78	24.3 \pm 12.96	0.87 \pm 0.54	9.6 \pm 4.95	0.75 \pm 0.34	2.7 \pm 0.66	4.6 \pm 0.48
Mean	26	21.3	784/447	2.5 \pm 0.35	23.6 \pm 0.99	0.9 \pm 0.02	8.5 \pm 1.55	0.9 \pm 0.15	3.0 \pm 0.45	4.7 \pm 0.21

Means are presented \pm s.d.
N, number of flies; T_a, ambient temperature.

mechanically applied pulses and the pressure response was below 1 ms for hemolymph pressure.

Measurements of intratracheal pressure

The intratracheal pressure was measured within the (meso)scutellar and abdominal air sacs. The cuticle was perforated and the air sac directly adhering to the inner cuticle was punctured (Fig. 1C). This procedure was performed after a few minutes of CO₂ anaesthesia to avoid damage and loss of hemolymph. The flies recuperated within minutes and spent up to the next 3 h grooming. The resting heartbeat or pressure cycles became obvious only when not obscured by motion effects. The resulting dorsal 'artificial spiracle' was tightly connected to a plastic cone (tip of an Eppendorf pipette with inner diameter of 1.6 mm, outer diameter of 2.3 mm) with Pattex glue (Henkel, Düsseldorf, Germany). The cone served as a holder. One or two polyethylene tubes (1 mm external diameter and 0.5 mm internal diameter) were inserted into the cone. The space around these tubes was tightly sealed using Fixogum. The adapter cone allowed the fly to be connected to the pressure sensor (Sensym SCXL 004 DN, Sensortechniques, Puchheim, Germany). The dead space of the external system of the pressure sensing system with a connecting tube of 48–81 mm length was approximately 10–16 µl. These differences in tube length had no measurable effect (delay or dampening of signal) when changing from shorter to longer tubes. The intratracheal pressure data were calibrated using an electronic calibration manometer (total scale ± 1 kPa, Manocal P, Besançon, France). The delay between mechanically applied pulses and the pressure response was approximately 1 ms for air pressure.

Data acquisition and analyses

Data were continuously recorded on an Apple PowerMac or PowerBook (Apple, Cupertino, CA, USA) 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 Hz. A software-integrated low-pass input filter was used to minimise noise in the electrophysiological measurements. An integrated band-pass filter allowed me to resolve pulses in the temperature recordings. A Student's *t*-test was used to determine the significance between means of the duration of forward and backward pulse periods, heartbeat frequency, hemolymph pressure amplitude, intratracheal pressure and the delay between the heart pulse and tracheal pressure pulse. The linear regression curves in supplementary material Fig. S1 were calculated using Excel (Microsoft Corporation, Redmond, WA, USA).

RESULTS

Analysis of pulse direction by thermistor measurements

The dorsal vessel of the fly consists of an abdominal heart tube with an enlarged anterior conical chamber and a narrow, more passive thoracic aorta. The dorsal vessel of intact and un-narcotised *C. vicina* exhibits a very regular rhythm of longer pulse periods (22.9 \pm 1.8 s) alternating with shorter pulse periods (7.4 \pm 4.4 s, *N*=12 flies, *n*=442 tested sequences, *t*-test for differences in mean periodic length, *P*<0.0001; Table 1, supplementary material Table S1). This periodicity was documented using different techniques. When a slight temperature excess was locally applied to the heart by a laser beam (T-method with heat marking), alternating heating and cooling temperature changes were recorded with two thermistors fixed on the tergites above the abdominal heart segments. A clear directional attribution of heart peristalsis was obtained: after projecting the laser at the posterior thorax, the shorter pulse periods were warming (Fig. 2A), indicating a backward transport. When the laser beam

was applied between the thermistors at the third and fourth heart segments (Fig. 2B), the temperature curves showed a reciprocal effect. At the anterior site (H3), the temperature increased during the longer pulse periods, and at the posterior site (H4) the temperature increased during the shorter pulse periods. As the temperature increase indicated that the hemolymph pulses must come from the heating site, it can clearly be deduced that the longer pulse periods represent the forward pulse periods and the shorter ones represent the backward pulse periods. Inversely, a warming effect resulted from the forward pulse periods when heat was applied caudally of both thermistors (Fig. 2C).

With the C-method, overall changes in temperature gave information about local hemolymph accumulation or reduction as a consequence of the periodic hemolymph shift, in addition to single pulse recording. At the second to fourth heart segments, periods with backward pulses led to a drastic overall temperature decrease owing to conductive cooling by the accumulating (hemolymph) mass and increasing density below the thermistor site. In the course of forward pulse periods, a re-warming occurred because of the decrease in hemolymph mass in the abdomen and compensatory expansion of the better-isolating air sacs (Fig. 3A). Thus, the phasic up and down level in the thermistor curves caused by the changes in thermal convection and conduction clearly reflects the pulse direction, even with a single thermistor. This is in accordance with the directional analysis of pulse periods by the T-method with heat-marked hemolymph.

When the thermistors themselves were heated as in the C-method, a higher temporal resolution of the single heart pulses was possible. Each bulk flow propelled by heart peristalsis produced a cooling effect upon the measuring thermistor with a re-warming during diastasis and beating pauses (Fig. 3). With two thermistors, it was possible to determine the pulse direction (Fig. 3C–F). The cooling effect represents the duration of hemolymph flow below the thermistor and is assumed to comprise the presystolic wave and a systole lasting approximately 80 to 110 ms per pulse. There was no difference in the duration of the cooling pulse of forward and backward pulses in the middle of the abdominal heart. The systole was likewise short during forward and backward pulses (forward: 86 \pm 20 ms; backward: 87 \pm 16 ms; *t*-test, *P*=0.7, *N*=7 flies, *n*=408 sequences; Fig. 3E,F). The difference in pulse rate between forward and backward pulses resulted from the different duration of the diastole, which was significantly longer during forward pulses (315 \pm 76 ms) than during backward pulses (155 \pm 36 ms; *t*-test, *P*=0.001).

Pulse pauses of up to several seconds (mean=0.9 \pm 0.15 s) occurred at the transition from forwards to backwards and *vice versa* (Fig. 3C,D, Table 1). Often, the last pulses of a forward pulse period were incomplete pulse waves, initiated at the same time from the anterior and posterior pacemakers, and collided at the level of the thermistor sites (Fig. 3D). The mean rate of forward pulses was significantly lower (3.4 \pm 0.45 Hz) than that of the backward pulses (4.9 \pm 0.57 Hz, *N*=12 flies, *n*=442 sequences; *t*-test for differences in mean heart rate, *P*<0.001; Table 1, Fig. 4). However, the pulse rate fluctuated during forward and backward pumping with a minimum frequency towards the end of the periods (Fig. 4E).

The speed of the travelling hemolymph wave over the distance of 2.1 mm between heart segments 3 and 4 ranged between 20 and 40 ms (105 and 52 mm s⁻¹, respectively) during forward pumping and between 17 and 30 ms (123 and 70 mm s⁻¹, respectively) during backward pumping, when comparing the time span of the cooling effect (Fig. 3E,F). The thermistor cooling effect represents both the hemolymph wave preceding the systole and the systole itself of the

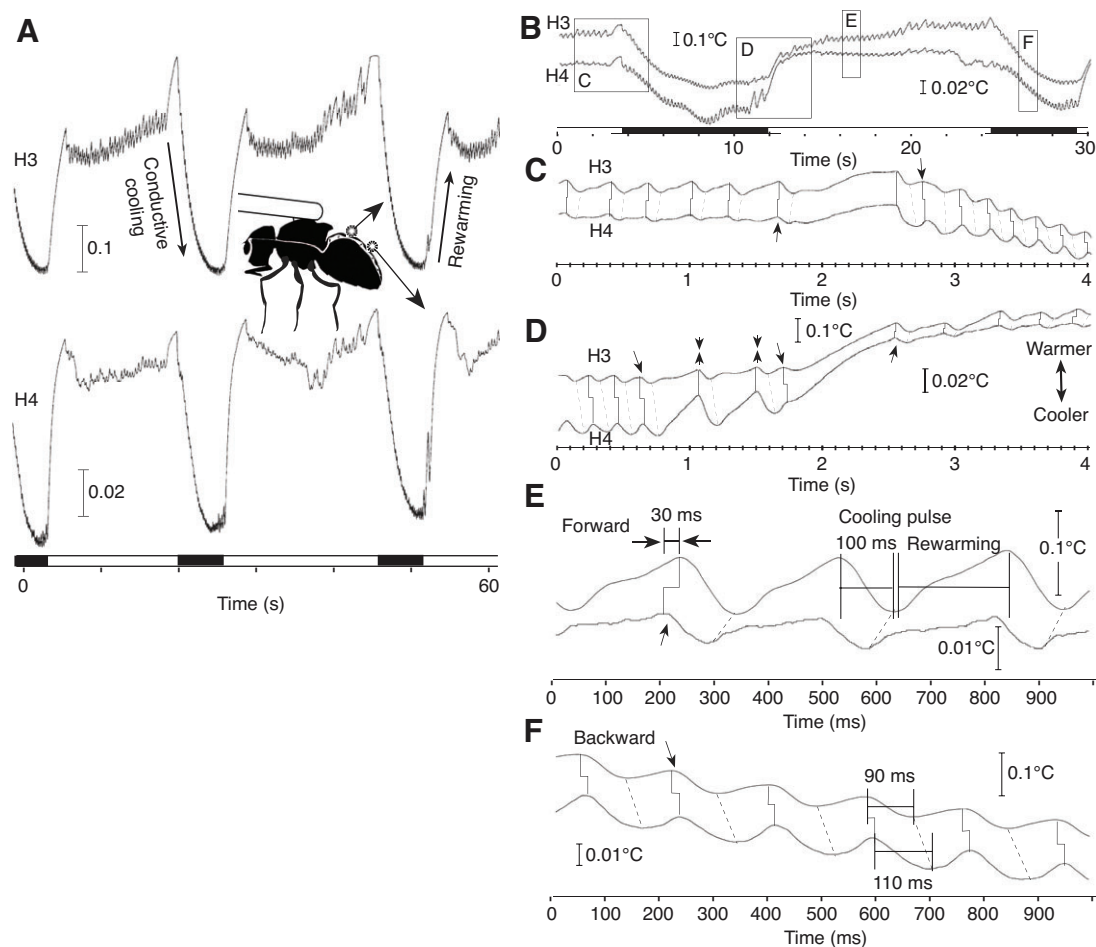


Fig. 3. Periodic changes in thermal convection and conduction due to hemolymph shift in the body by heartbeat reversals in intact resting *C. vicina* (C-method). (A) Thermistors heated by ΔT 1.8°C at tergites 3 and 4. The temperature decrease at the heated thermistor sites indicates an increase of convective and conductive cooling by augmentation of hemolymph in the abdomen during backward hemolymph transport (black bars). Temperature increase without pulses indicates pauses during transition from forward to backward and from backward to forward pulse periods. Ambient temperature (T_a)=25°C. (B–F) Determination of pulse direction and velocity by the timing of convective effects of heart pulses at segments H3 and H4. Each convective cooling event corresponds to peristalsis-induced hemolymph flow. (B) Sequence of forward and backward pulse periods. (C) Transition from forward to backward beating, detail from B. (D) Transition from backward to forward beating, detail from B. At the end of the backward pulse period, two collisions occur (double arrows). (E) Three forward pulses per second, detail from B. (F) Six backward pulses per second, detail from B. T_a =25°C. Arrows in C–F point to the directional change of pulses.

heart pulse. The presystolic bulk flow results in a higher speed than the progression of the electrophysiologically recorded systole. The velocity of the systolic wave was therefore analysed and statistically evaluated on the basis of the electrophysiological measurements (see below).

Heartbeat analysis confirmed by electrical resistance measurements

In an earlier study on the heart of *Protophormia terraenovae*, it was argued that the ‘fast phases’ are forward and the ‘slow phases’ are backward, explaining the inverse attribution of the pulse direction on the basis of thermistor measurements being less conclusive because of the indirect method (Angioy et al., 1999). To confirm the results from the thermistor records in the present study, a comparison with a more direct electrophysiological technique was performed. The duration of pulse periods, pulse direction and pulse rate corresponded to the thermistor data (Table 1, Fig. 4A–C, supplemental material Table S1): the forward pulses were longer and exhibited a lower pulse rate than the backward pulses. In

addition, the forward pulses were more complex, with more than one peak in contrast to the very regular and uniform backward pulses (Figs 5, 6). With two paired electrodes implanted at the second and the fourth heart segments, the systolic progression was measured over a distance of 3.5 mm (longer than in the thermistor records). The velocity of the forward pulses was $36 \pm 4.6 \text{ mm s}^{-1}$, slower than the backward pulses at $60 \pm 8.1 \text{ mm s}^{-1}$ ($N=4$ flies, $n=65$ sequences; Fig. 4D). The higher velocity of the backward pulses, measured with both methods, is one reason why the backward pulses result in a higher pulse rate.

Heartbeat reversals and hemolymph pressure in the anterior body

Periodic changes in heartbeat direction result in a hemolymph shift between the anterior body and the abdomen. It is a question of how the periodic volume changes affect the pressure in the anterior body with a more sclerotised integument and the abdomen with a more compliant exoskeleton and intersegmental muscles. As expected, the shifting of the hemolymph caused alternating pressure changes

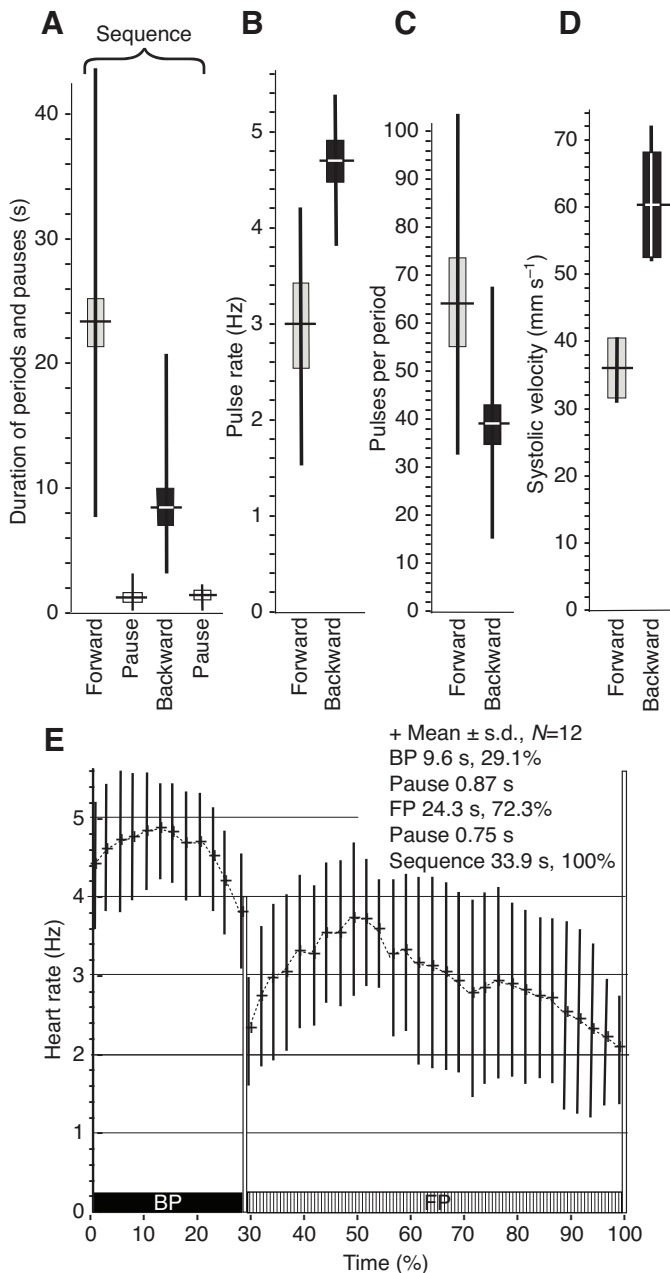


Fig. 4. Heart activity of *C. vicina* during forward and backward pulse periods. Data are means \pm s.d.; vertical lines indicate the range, and the boxes enclose \pm s.d. (A) The duration of forward pulse periods was always longer than that of backward pulse periods. Between the pulse periods, pauses of approximately 1 s appear. (B) Mean pulse rate was lower during forward beating than during backward beating. (C) Number of pulses per period. (D) Systolic velocity was measured by electrodes along the distance between heart segments 3 and 4. The pulse was quicker in the backward direction than in the forward direction. Data in A–C are based on Table 1 and supplementary material Table S1, $N=26$ flies, evaluated sequences: mean=30.6 sequences per fly. Data in D are based on $N=4$ flies, $n=65$ sequences, $T_a=23\pm1^\circ\text{C}$. (E) Characteristic frequency fluctuations within heartbeat periods of 12 flies. Mean values \pm s.d. of three sequences per individual. FP, forward pulse period; BP, backward pulse period.

in the hemocoel of the thorax (Fig. 6, see Fig. 8A, Fig. 10). The pressure was measured at the punctured mesonotum, where a relatively large hemocoelic space lies medially under the cuticle between the dorso-longitudinal flight muscles. This allowed the

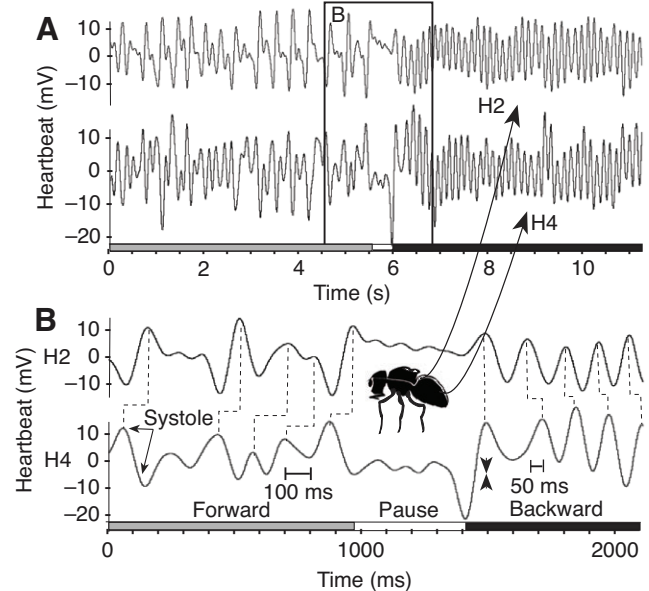


Fig. 5. Measurements of heartbeat in *C. vicina* with paired electrodes at the second (H2) and fourth heart segments (H4). (A) Change from forward to backward beating. (B) Detail from before and after the heartbeat pause. The origin of weak undulations during the heartbeat pause is unclear. The beginnings of systoles (downstrokes) are connected by stippled lines.

hemocoel to be connected with the tip of the saline-containing syringe needle directly at the surface without destroying the muscles. In the resting fly, after some hours of recovery from saline surplus, a slightly negative hemocoelic pressure became established between approximately -10 and -50 Pa (mean= -27 ± 15 Pa, $N=10$ flies; Table 2, supplementary material Table S2). This pressure always decreased further during backward beating to a lower pressure (mean= -242 ± 136 Pa, minimum= -774 Pa, $N=10$ flies, 1818 tested sequences; Fig. 8D). The pressure decreased in the course of the backward pulse period at a mean rate of approximately 12 ± 9 Pa s^{-1} ($N=10$ flies; Fig. 6, supplementary material Fig. S1). The complex forward heart pulses of the electrophysiological measurements generally preceded the positive hemolymph pressure pulses and the backward pulses preceded the negative pressure pulses (Fig. 6C, stippled lines: time interval between systole and pressure pulse). The positive pressure pulses had a significantly higher amplitude (14 ± 14 Δ Pa) than the negative pressure pulses (4.6 ± 5.3 Δ Pa, $N=10$ flies, each $n=20$ periods; t -test, $P<0.05$). Towards the end of a forward pulse period, only the pulses with high amplitudes were expressed in the pressure curves (Fig. 6B), or additional pressure pulses without corresponding heart pulses occurred (Fig. 7A). These irregularities suggest that an additional muscle system is involved in pressure pulse generation, which will be analysed in a subsequent paper.

Intratracheal pressure compared with hemolymph pressure in the anterior body

The anterior body contains an extended tracheal system with large elastic air sacs. In contrast to the mostly stiff exoskeleton, the compliance of the air sacs can compensate for changes in hemolymph volume. The question arises as to whether the positive and negative hemocoelic pressure pulses influence the tracheal pressure or whether the tracheal pressure is equilibrated with the ambient pressure through the spiracles. Periodic changes in

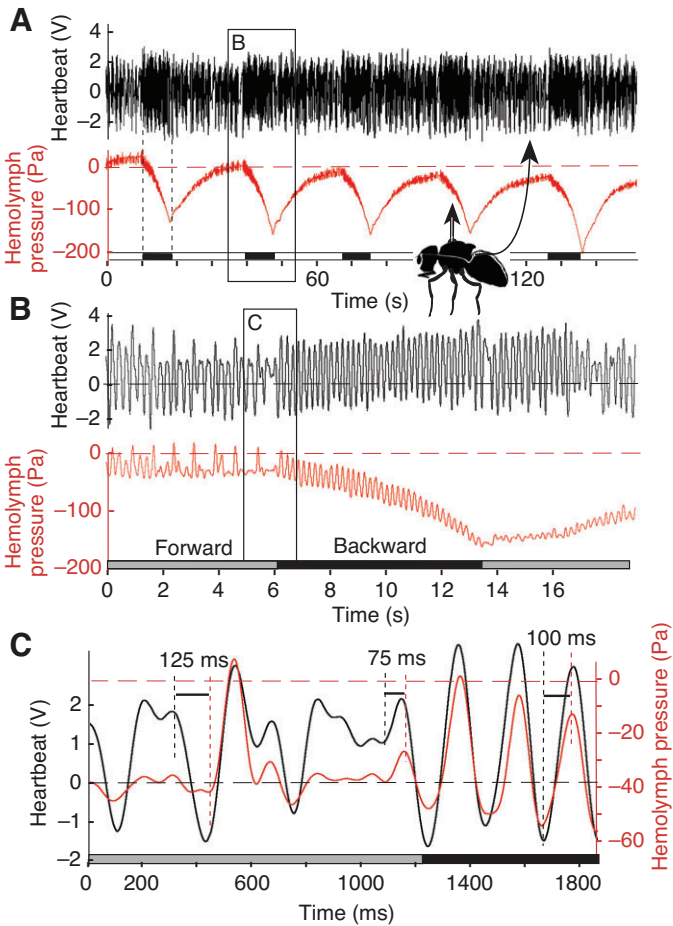


Fig. 6. Mesonotal hemocoelic pressure (red curves) and simultaneous electrophysiological recordings of the anterior heart chamber in a female *C. vicina*. Coordination of backward pulse periods (black bars) with periodically decreasing hemolymph pressure in the thorax. (A) Five representative sequences. (B) Detail from A. The forward pulses are more complex and irregular. Not all forward pulses are accompanied by a corresponding strong pressure pulse. (C) Detail from B. Transition from forward to backward pulses indicating the delay between heart pulse and pressure pulse. Pulses at the transition from forward to backward beating reveal that all heart pulses precede the pressure pulses. The systole (downstroke) of the forward pulse is followed by a positive pressure pulse (upstroke) of the pressure curve. The diastole (upstroke) of the backward pulse is followed by a negative pressure pulse (heart pulses are connected with the corresponding pressure effect by a stippled line).

intratracheal pressure pulses were obvious and regular in flies when they had calmed down ($N=17$ flies; Table 2, supplementary material Table S3). Under stress and directly after preparation and CO_2 anaesthesia, spiracles were observed to be fully open. Intratracheal pressure changes were then immediately equilibrated to the

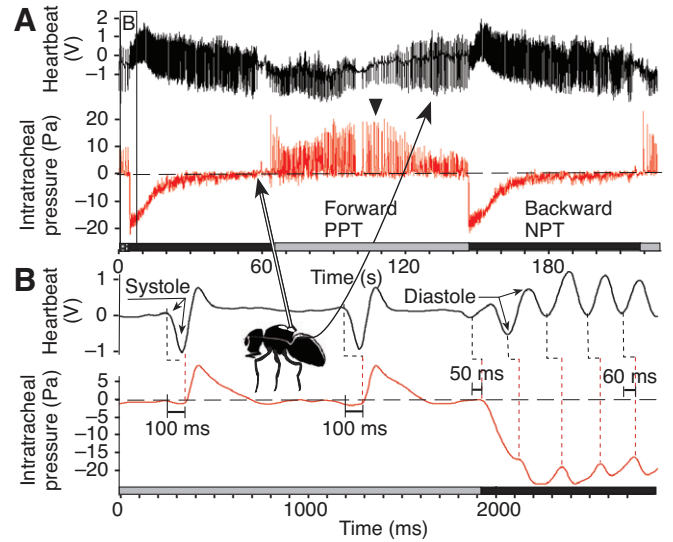


Fig. 7. Intratracheal pressure of the scutellar air sac and simultaneous electrophysiological recordings of the heartbeat. (A) Survey. Pressure pulses correspond to heart pulses, except some pressure pulses during forward pulse periods with high amplitude (arrowhead). (B) Detail of A. The interval between the backward pulse and the pressure pulse is shorter than the interval between the forward pulse and the pressure pulse. Heart pulses are connected with the corresponding pressure effect by a stippled line. Explanation as in Fig. 6.

atmospheric pressure. The re-establishment of the intratracheal pressure pattern depends on the partially closing of the spiracles. In the thoracic air sacs of motionless resting flies, shorter periods with negative pressure pulses (NPTs) alternated with longer periods with positive pressure pulses (PPTs). The pulses generally coincided with the heart pulses and the pulse pressure reflects the direction of heart peristalsis (Figs 7, 8); if the heart pulsed forwards, the pressure pulses were positive and had a high amplitude. The first pulses of each PPT often had the highest amplitude, diminishing over the course of the period. Towards the end of each PPT, the pulses became less frequent and more irregular, like the heartbeat. The first negative pulses of the NPTs caused a steep pressure decrease. In the course of the NPTs, the mean pressure increased from more negative to less negative, although the single backward pulses retained the same amplitude. The NPTs were much more regular than the PPTs during forward heartbeats. The delay between the electrophysiological signal of the heart pulse and the beginning of the corresponding pressure pulse in the scutellar air sac was different in both directions. The delay was significantly longer during forward pulses (92 ± 15 ms) than during backward pulses (61 ± 19 ms, $N=7$ flies, 91 forward pulses and 89 backward pulses per fly were evaluated; t -test, $P<0.004$). To what extent is the hemocoelic pressure transmitted to the tracheal system? The mean thoracic

Table 2. Pressure in the thoracic hemocoel and scutellar air sac of *Calliphora vicina*

	<i>N</i>	<i>T_a</i> (°C)	Sequences		Pressure (Pa)			Pulse amplitude (ΔPa)	
			No./min	No. min ⁻¹	Mean upper value during PPT	Mean lower value during NPT	Lowest value during NPT	PPT	NPT
Thoracic hemocoel	10	21.5	1818/1078	1.5±0.7	-27±15	-242±136	-774	14±14	4.6±5.3
Scutellar air sac	17	21	1645/1005	1.9±0.9	8.8±13	-8.1±17	-85	10±9	3.0±2.5

Means are presented ±s.d.
N, number of flies; NPT, negative pressure pulse periods; PPT, positive pressure pulse periods; *T_a*, ambient temperature.

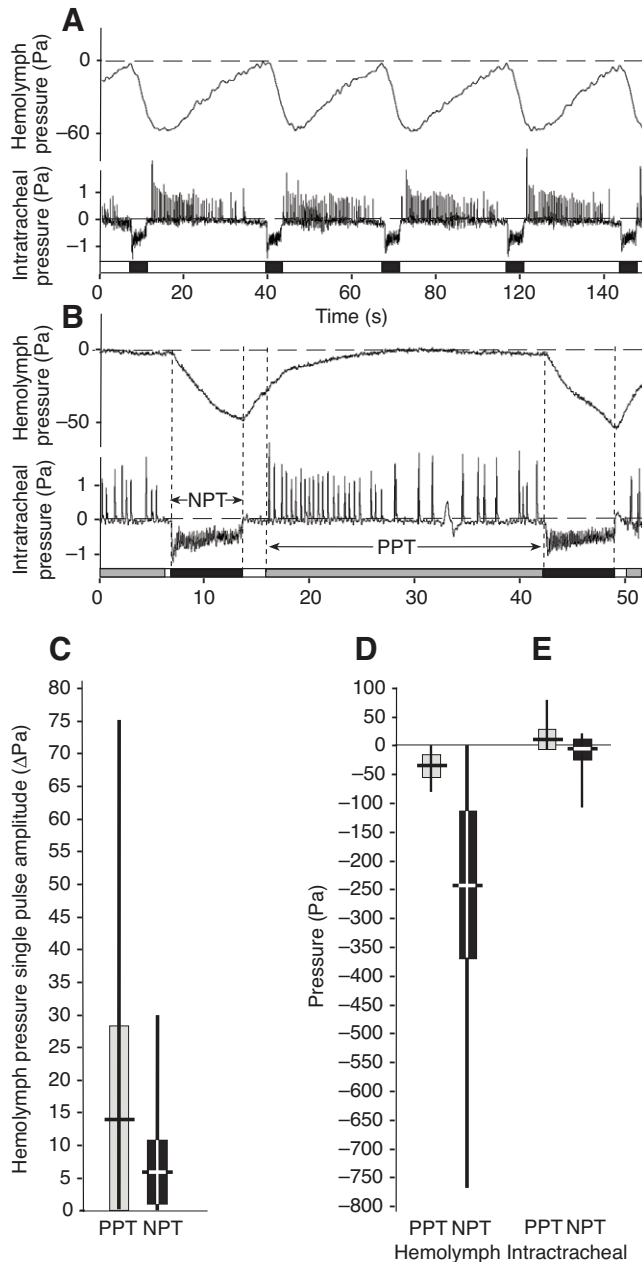


Fig. 8. Comparison of hemolymph and intratracheal pressure measured in the mesonotal hemocoel and scutellar air sacs, respectively. (A) Simultaneous recordings of hemolymph pressure (upper trace) and intratracheal pressure (lower trace). (B) Curves with higher resolution. In the hemocoel, the pressure is permanently sub-atmospheric and always decreases gradually during negative pulse periods (NPTs). By contrast, intratracheal pressure drops abruptly to the lowest sub-atmospheric value with the first negative pulses, then increases slowly and becomes supra-atmospheric by the positive pressure pulses (PPTs). Black bars indicate backward pulse periods of the heart. (C–E) Hemolymph pressure and intratracheal pressure in the thorax. Data are means \pm s.d.; vertical lines indicate the range, and the boxes enclose \pm s.d. (C) The amplitude of the pressure pulses in the hemocoel is very variable. The pulses during PPTs have a higher amplitude than those of the NPTs. (D) The mean hemolymph pressure during PPTs is slightly sub-atmospheric with values of -27 ± 15 Pa. During NPTs, the negative pulses make the mean hemolymph pressure more sub-atmospheric at -242 ± 136 Pa with extreme values of approximately -774 Pa. Data in C and D are from Table 2 ($N=10$ flies, 1818 evaluated sequences). (E) Supra-atmospheric intratracheal pressure during PPTs and sub-atmospheric pressure during NPTs. Data are from Table 2 ($N=17$ flies, 1645 evaluated sequences).

hemocoelic pressure in *C. vicina* was always sub-atmospheric. By contrast, the tracheal pressure oscillated around ambient pressure with positive pulses during forward pulse periods and a significantly higher pressure ($+8.8 \pm 13$ Pa) than that during backward pulse periods, with negative pulses and a negative mean pressure level (-8.1 ± 17 Pa, $N=17$ flies, 1645 evaluated sequences, t -test $P < 0.01$; Fig. 8, Table 2, supplementary material Table S3).

Coordination of abdominal movements with heartbeat reversals

In contrast to the thorax, the abdomen is capable of actively changing its volume; however, it is unclear whether it only reacts passively to changes in hemolymph volume or whether active changes in muscle tension assist in sucking or pressing. During heartbeat reversal, the abdomen performed coordinated movements ($N=8$; Fig. 9A, supplementary material Movie 1). It prolonged and enlarged slightly at the level of tergites and sternites 3 and 4 during backward beating. During forward beating, it shortened and reduced the distance between tergites and sternites 3 and 4. Immediately at the beginning of the forward pulses, the posterior abdominal segments 5 to 7 showed a single slow pumping stroke, which can be seen as a peak and a following slope in the curves (arrowheads, Fig. 9B) and as a contraction in supplementary material Movie 1. It also affected the pressure curves of the abdominal hemocoel (Fig. 10) and air sacs (Fig. 11). This contraction was not detectable in the pressure sequences of the thorax. During heart pauses, no abdominal movements occurred.

Hemolymph and air sac pressure in the abdomen

To determine whether heartbeat reversals and abdominal movements, especially the pumping stroke, exert some influence on pressure, hemolymph and tracheal pressure was measured. The hemolymph pressure in the abdomen reflects the periodic accumulation and drainage of the hemolymph caused by the hemolymph shift, which is inverse to the thorax ($N=5$ flies, 155–160 evaluated sequences per fly). The pressure curves also show the superimposed periodic activity of the abdominal wall muscles. At the beginning of the forward pulse period, a pressure peak with a following pressure decrease (arrowheads, Fig. 10) was attributed to the pumping stroke (Fig. 9B). Another weak pressure peak sometimes occurred at the beginning of the backward pulse periods (asterisk, Fig. 10). The pressure in the abdominal hemocoel attained the highest positive values between $+80$ and $+220$ Pa (mean $= +80.4 \pm 81$ Pa, $N=5$) during the contraction peak at the beginning of the forward pulse period and the lowest negative values (mean $= -191 \pm 120$ Pa) at the end of the forward pulse periods with the lowest values at approximately -330 Pa. During backward pulse periods, the pressure in the abdomen started at a negative level and increased to a mean of 10 ± 8 Pa above atmospheric pressure towards the end.

Pressure effects during heartbeat reversals in the abdominal air sac were also opposite to those in the thorax, reflecting the changes in hemolymph volume. During backward pumping of the heart, the pulses were positive (amplitude $= 0.07 \pm 0.006$ Δ Pa) and the mean pressure was slightly above atmospheric. It decreased during forward pumping by negative pressure pulses (amplitude $= 0.18 \pm 0.03$ Δ Pa, $N=3$ flies, 566–570 evaluated sequences per fly). The positive pressure peak ($+1.2 \pm 0.5$ Pa, with a maximum of $+2$ Pa) and the following abrupt pressure decrease (-5 ± 3 Pa, with a minimum of -12 Pa) during forward pulses are attributed to the abdominal pumping stroke (arrowheads, Fig. 11). The negative forward pulses are superimposed on the pumping stroke.

Heartbeat reversal proved to be the major mechanism of air sac pressure pulses alternating in the anterior and posterior body. The

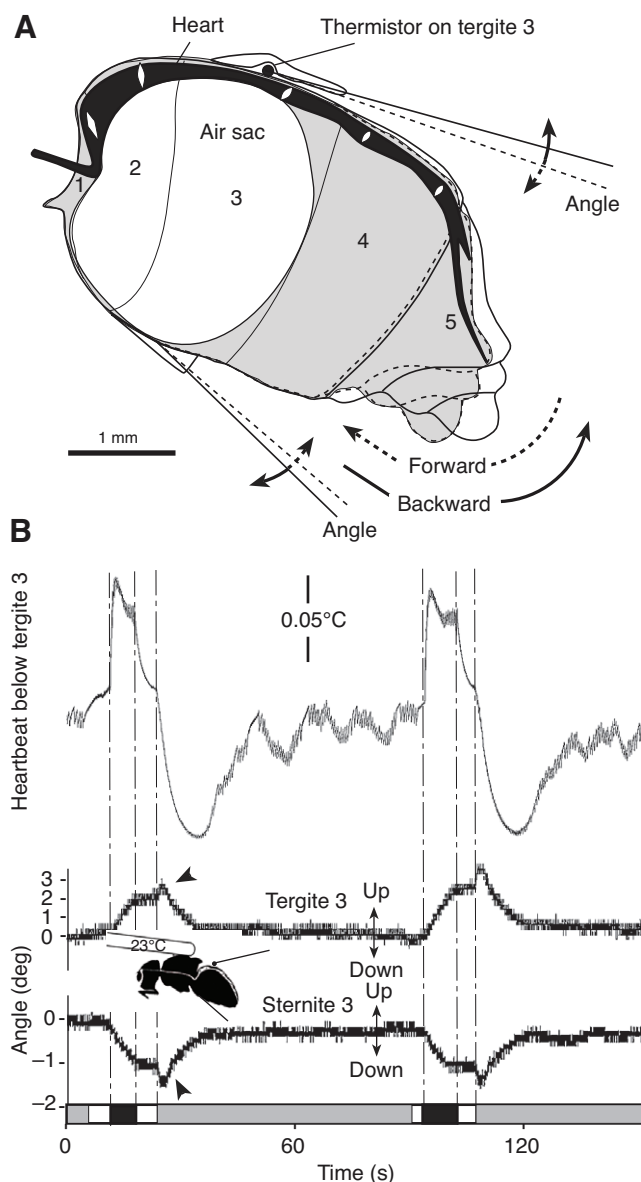


Fig. 9. (A) Changing contours of the abdomen during a heartbeat sequence, based on video frames (supplementary material Movie 1). The slow downwards bending of the abdominal tip is combined with a reduction in the diameter of the abdominal segments. The corresponding reduction or increase in volume coincides with forward (stippled lines) and backward beating, respectively, as recorded in B. (B) Coordination of tergo-abdominal and sterno-abdominal movements with heartbeat periodicity. Grey bars, forward heartbeat; black bars, backward heartbeat; white bars, pause; arrowheads, pumping stroke.

periodic changes in mean pressure reflect the forward and backward pulse periods. NPAs correspond to forward pulse periods and PPAs correspond to backward pulse periods of the heart in the abdominal air sacs (Fig. 11), and *vice versa* in the thoracic air sacs.

DISCUSSION

Regular periodic heartbeat reversals – controversy about the directional attribution of peristalsis is settled

Heartbeat reversal in flies has been described several times. In adult flies, the effects of diverse external and internal stimuli on heartbeat reversals have been studied, e.g. olfactory stimuli (Campan and Queinnec, 1972), visual stimuli (Thon, 1980; Thon, 1982), sexual

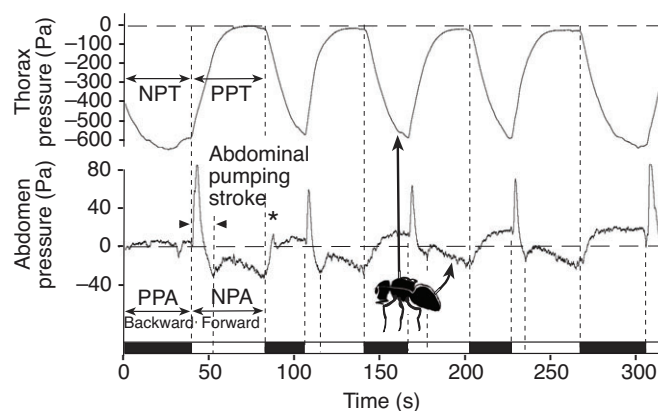


Fig. 10. Simultaneous recordings of hemolymph pressure in mesothorax and in abdominal segment 4. In the thorax, the pressure reflects the periodic inflow and outflow of hemolymph according to heartbeat reversal. Here, the backward pulses are measured as negative pulses (NPT). In the abdomen, they are measured as positive pulses (PPA). During backward pulse periods, the abdominal pressure increases, while during forward pulse periods it decreases. However, at the beginning of the forward pulse periods, a positive pressure peak results from the abdominal pumping stroke (indicated between arrowheads). NPA, negative pulses in the abdomen.

maturity (Queinnec and Campan, 1975) and food stimuli (Angioy, 1988). Pulse direction has been interpreted controversially. Most authors attributed the higher pulse rate to the forward heartbeat (also 'anterograde' or 'fast phase') and the lower pulse rate to the backward heartbeat (also 'retrograde' or 'slow phase') (Brazeau and Campan, 1970; Queinnec and Campan, 1975; Thon and Queinnec, 1976; Thon, 1982; Angioy and Pietra, 1995; Dulcis and Levine, 2005; Slama and Farkás, 2005; Slama, 2010) as it is the case in *Lepidoptera* (Gerould, 1929; Wasserthal, 1980; Wasserthal, 1981; Hetz et al., 1999; Smits et al., 2000; Slama and Miller, 2001). However, the analysis with thermistor measurements under heat marking in intact flies suggested an opposite frequency attribution (Wasserthal, 1982b; Wasserthal, 1999). In mosquitoes, as in *C. vicina*, the longer pulse periods correspond to the forward pulse direction. However, in mosquitoes the pulse rates of forward and backward direction are not different (Glenn et al., 2010). The uniformity in the mosquito heart rate may result from a more ancestral hemolymph transport, e.g. by the existence of an abdominal perineural sinus (Richards, 1963). In the present study, the heartbeat was analysed using thermistor and electrophysiological measurements. Both methods revealed that the shorter periods with the higher heart rate are backwards, confirming the analysis in intact *D. melanogaster* and *D. hydei* (Wasserthal, 2007) and challenging the interpretation of the authors cited above.

Further support for the present diagnosis of pulse direction is provided by the pressure measurements in the thoracic and abdominal hemocoel and air sacs. The positive pulses lead to an increase of hemolymph pressure in the anterior body, corresponding to the measured forward pulses. The negative pressure pulses lead to a decrease in pressure in the anterior body, representing the expected effect of the backward pulses. The pulse rate during each pulse period was not constant, but fluctuated in a typical way, being higher at the onset and lower at the end. Thus, the forward pulses right at the beginning could have a higher frequency than the pulses at the end of a backward pulse period. Comparison of the last backward pulses with the first forward pulses might have given the impression that the forward pulses had a higher pulse rate. However,

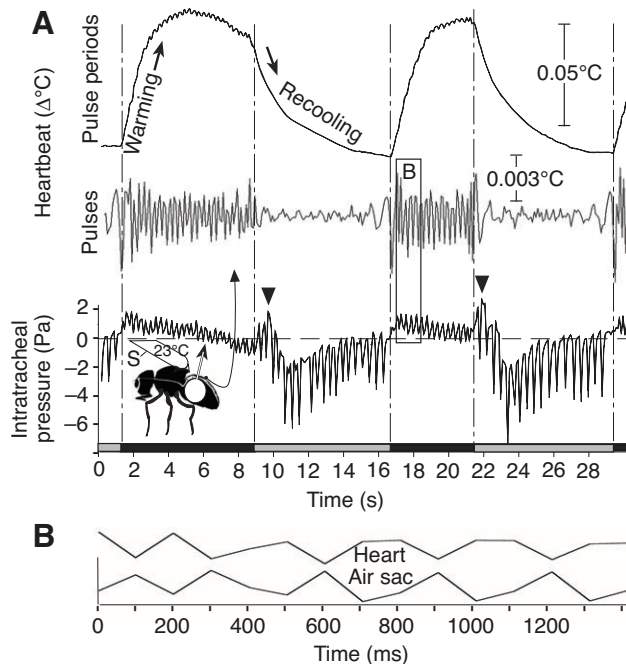


Fig. 11. Abdominal air sac pressure and concurrent periodicity of heartbeat at heart segment 3 in a female *C. vicina*. (A) Upper trace: T-method under heat marking of the thoracic hemolymph with a soldering bit (S), ΔT of 2°C above $T_a=21^{\circ}\text{C}$, which heat marks the backward pulses of the heart. Middle trace: heart pulses of the upper trace (band-pass filter 20–0.5 Hz). Lower trace: intratracheal air sac pressure. During backward beating, positive pulses augment the tracheal pressure above ambient pressure. Pressure peaks are a reaction to the single abdominal pumping strokes (arrowheads). This is followed by negative pressure pulses, reflecting the hemolymph reduction in the abdomen by forward pulses. Black bars, backward pulse periods; grey bars, forward pulse periods. (B) Detail of A: coincidence of heating moments of backward pulses with negative moments of pressure pulses in the air sac. Although the mean pressure during backward pulses is positive, the single pressure pulses look negative, which is probably caused by the contraction of the conical heart chamber pulling the neighbouring air sac walls up and out, which increases the volume of the air sacs without delay.

when comparing the mean frequency during the entire period, the results are in favour of attributing the higher pulse rate to the shorter periods with the backward pulses. The velocity of the backward pulses was also higher than that of the forward pulses according to the relative speed difference in most *D. hydei* (Wasserthal, 2007).

A pumping-suction mechanism: an analogy for heart and abdomen function

The periodic heartbeat reversals seem to cause the periodic pressure changes in the hemocoel and tracheal system. Videos and measurements showed that the abdomen expands, facilitating hemolymph release through the caudal excurrent openings during backward beating of the heart. Lacking a valve structure at the excurrent opening (Angioy et al., 1999; Wasserthal, 1999), hemolymph, leaving the caudal opening during systole of the backward pulses, might be sucked back into the heart during the following diastole via the inflow ostia, resulting in a standing wave. The negative hemolymph pressure in the abdomen at the beginning of the backward pulse period might imply a suction effect, supporting the depletion of the heart (Fig. 10). However, during the following backward pulses, the abdominal hemocoelic pressure increased and became even slightly supra-atmospheric. It is probable that, under

these conditions, hemolymph reflux into the heart during diastole is prevented by the presystolic wave of the following backward pulse refilling the diastolic posterior heart. The recorded abdominal volume increase could then be interpreted as a relaxation with a change in intersegmental muscle tonus. The cushion-like sarcoplasmic swellings, narrowing the posterior heart lumen during contraction (Wasserthal, 1999), may prevent a reflux inside the heart and thus contribute to an efficient discharge of hemolymph in both directions.

This mechanism is partly reminiscent of the system in the giant silk moth, where the abdomen lengthens during backward pulses and contracts during forward pulses (Wasserthal, 1981). In addition, the moth abdomen reacts with peristaltic ventilatory movements at the end of the backward pulse periods, the moment of maximal accumulation of hemolymph in the abdomen (Wasserthal, 1976; Wasserthal, 1981). In *C. vicina*, a single pumping stroke generally coincides with the onset of the forward pulse periods, when the hemolymph is accumulated in the abdomen. This stroke is perceived in the pressure curves of the abdominal hemocoel and air sacs. It has no distinct effect on the pressure in the anterior body (Fig. 11). The tracheal systems of the anterior and the posterior body are not connected because of a degeneration of the longitudinal trunks in the metathorax (Faucheux, 1973). It is assumed that the pumping stroke, with a slow hemocoelic pressure increase at the beginning of the forward pulse period, supports an efficient anterograde transport by the heart in flies, as in adult moths (Wasserthal, 1996). The ventilatory bouts in moths and the single pumping stroke in this fly are superimposed on the slow volume changes. The coincidence of abdominal ventilatory movements with certain phases of the heartbeat has also been reported from lepidopteran and coleopteran pupae (Tartes et al., 1999; Tartes et al., 2000; Tartes et al., 2002). Experimental prevention of abdominal movements in mosquitoes (Jones, 1954) and in *D. melanogaster* (Wasserthal, 2007) caused the heart to stop or to beat erratically.

Measurement of hemolymph pressure in small insects over longer periods is a challenge requiring delicate techniques. The first pressure measurements in flies and Lepidoptera were performed for analysis of the eclosion process and revealed positive pressures necessary for eclosion and wing inflation (Cottrell, 1962; Moreau, 1974; Slama, 1976). The hemocoelic pressure data of pupae and adult insects commonly show negative pressures ranging between -20 and -85 Pa in the puparium of the flesh fly *Sarcophaga bullata* (Slama, 1984) and between -210 and -1150 Pa in the pupae of Lepidoptera (Slama, 1984). Attribution of pressure pulses to the responsible muscle system is difficult, as all muscles inside the open hemolymph system principally exert some influence on hemocoelic pressure. Extracardiac pulses or coelopulses in pupae have been attributed to intersegmental abdominal muscles (Slama, 1988). In the present study, simultaneous electrophysiological measurements of heart pulses and pressure in the blowfly showed for the first time that thoracic pressure pulses in *C. vicina* generally coincide with the pulses of the heart and not with the intersegmental muscles of the abdomen, which in these flies move at a much lower frequency, i.e. one contraction per sequence of heartbeat reversals. Additional pulses in the pressure curves or slower superimposed pressure pulses below the heartbeat frequency measured in the anterior body hint at the involvement of additional pulsatile systems in the anterior body, which will be dealt with in a subsequent paper.

Thoracic hemocoelic pressure is generally lower than intratracheal pressure

The mean thoracic air sac pressure in the present study was revealed to be partially different from thoracic hemolymph pressure: single

positive pulses and the negative intratracheal pressure pulses generally corresponded to the positive and negative hemocoelic pressure pulses. However, even when the intratracheal pressure in the thorax exhibited positive pulses above atmospheric pressure, caused by heart pulses, the mean pressure in the thoracic hemocoel still remained subatmospheric (Fig. 8A,B). Hemocoelic pressure is determined by the small hemolymph volume (Brocher, 1931; Jones, 1977), enclosed in a sclerotised integument with only partly compliant intersegmental membranes. Most compliance comes from the elastic walls of the air sacs. They are set under permanent tension after the post-ecdysial diuretic process (Cottrell, 1962; Nicolson, 1976). If a forward heart pulse dilates the aorta and augments the hemolymph volume in the anterior body, the volume of the air sacs is reduced correspondingly, but the tension of the tracheal walls is never fully lost. Thus, the hemolymph is maintained under negative pressure. This might be the reason why the intratracheal pressure is not a mere reflection of hemocoelic pressure. In the thoracic tracheal system, the lowest scutellar air sac pressure arises at the beginning of the backward pulses, whereas in the thoracic hemocoel, the pressure decreases gradually towards the end of the backward pulses (Fig. 7A, Fig. 8A). Inversely, during forward pulses, when the first intratracheal pressure pulses show the highest amplitudes, pressure in the thoracic hemocoel increases only gradually (Fig. 8A). This suggests that the tension of the tracheal system is only partly abolished by the volume change due to hemolymph accumulation. The compliance of the integument and tracheal system gives way only partly to the changing hemocoelic pressure. The increased tension of the distended thoracic and cephalic tracheal system at the end of the backward pulse periods may support hemolymph transport during the following forward pulses of the heart. This and the abdominal contraction may explain why the pulse amplitude of the first forward pulses is higher than that of the last pulses of the forward pulse period. When the supportive suction effect due to the relaxing air sacs becomes weaker at the end of the forward pulse period, the pulse amplitude and heart rate become reduced. It is probable that the heart has to work against a higher resistance at the end of the pulse periods, receiving less hemolymph from the 'drained' compartment.

Conclusions

The periodic hemolymph shift in *C. vicina* by heartbeat reversal has been revealed to be a major mechanism for maximising the hydraulic efficiency of a small hemolymph volume. The separation of the fly's body into anterior and abdominal compartments allows the use of a small amount of hemolymph for efficient pressure production by a pumping-suction mechanism. The pressure of the tracheal system depends on the hemolymph pressure, but it is not identical, owing to its communication with the ambient air *via* spiracles. The influence of the periodic pressure changes on the tracheal volume and respiratory gas exchange will be investigated in a separate publication.

LIST OF ABBREVIATIONS

IR	infrared
NPA	negative pressure pulse period in the abdomen
NPT	negative pressure pulse period in the thorax
PPA	positive pressure pulse period in the hemocoel or air sacs of the abdomen
PPT	positive pressure pulse period in the hemocoel or air sacs of the thorax
RCD	reflex coupling device

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Table S1. Heartbeat frequency and duration of sequences, pulse periods and pauses, based on thermistor and electrophysiological recordings

Specimen ID/Year	T_a (°C)	Sequences		Duration of pulse periods and pauses (s)				Pulse rate (Hz)	
		No./min	No. min ⁻¹	Forward	Pause	Backward	Pause	Forward	Backward
Thermistor recordings									
M2/1994	20	36/25	1.44	25.2±2.7	2.9±1.4	12.6±2.9	1.0±0.4	2.6±0.3	3.8±0.2
F1/1995	20	32/10	3.2	10.5±1.6	0.6±0.5	6.3±1.4	1.4±1.1	3.4±0.1	5.1±0.5
F2/1995	20	31/8	3.87	7.6±2.3	0.5±0.16	5.1±0.6	0.9±0.4	3.8±0.5	4.8±1.3
F3/1995	20	32/9	3.5	10.5±1.5	0.5±0.05	4.8±0.2	1.0±0.5	3.4±0.2	4.8±0.2
F4/1995	20	30/11	2.72	12.6±2.1	1.0±0.3	6.2±0.7	2.2±0.7	3.0±0.2	5.1±0.3
F2/2000	20.5	33/19	1.73	24.3±4.9	2.0±0.65	5.7±2.5	2.1±0.4	3.2±0.3	3.7±0.1
M2/2007	21	31/15	2.06	20.0±7.1	0.6±0.3	8.5±6.3	0.2±0.1	3.2±0.3	5.1±0.2
M8/2007	20.3	90/32	2.81	17.2±3.6	0.3±0.1	3.5±0.6	0.2±0.1	4.2±0.2	5.2±1.1
F9/2007	21.8	32/31	1.03	37.3±7.7	1.5±1.28	18.1±9.8	1.0±0.3	3.3±0.2	5.3±0.1
F1/2008	21.7	32/46	0.69	74.0±21	0.1±0.05	10.6±7.0	0.8±0.3	3.9±0.1	5.1±0.3
F2/2008	21.7	32/12	2.66	17.0±5.8	0.6±0.32	3.4±0.9	0.4±0.2	4.0±0.5	5.4±0.3
F3/2008	21	31/13	2.38	19.3±10	0.3±0.1	4.2±1.3	0.5±0.1	3.7±0.2	5.4±0.1
Electrophysiological recordings									
F10/2008	21.5	32/15	2.13	19.0±6.5	0.2±0.16	7.8±6.1	0.2±0.1	3.7±0.3	4.8±0.1
M15/2009	22	35/25	1.45	25.7±5.1	0.6±0.32	14.6±5.9	0.8±0.2	2.5±0.2	5.0±0.3
M2/2009	22	10/4	2.5	9.37±2.2	0.5±0.46	6.4±3.32	0.9±0.4	4.0±0.46	5.2±0.55
M3/2009	21.5	39/33	1.18	33.0±13.3	1.1±0.37	13.7±4.16	0.6±0.17	3.2±0.5	4.7±0.5
M4/2009	22	5/7	0.7	59.4±14.9	1.3±0.49	21.5±6.9	1.2±0.44	2.5±0.2	4.2±0.83
F5/2009	22	20/7	2.8	16.3±2.8	0.3±0.14	4.9±1.79	0.13±0.08	2.6±0.4	4.0±0.46
F23/2009	22.5	32/12	2.6	13.1±2.5	1.7±0.69	5.0±0.64	0.59±0.10	1.9±0.25	5.1±0.21
F13/2009	21	5/4	1.25	31.3±9.0	0.36±0.13	10.9±5.87	1.0±0.37	2.5±0.67	4.4±0.39
F12/2009	21	35/16	2.18	19.6±8.68	0.52±0.34	8.0±3.81	0.7±0.22	3.2±0.33	5.0±0.33
F11/2009	21	24/18	1.33	25.3±6.81	1.9±1.28	15.1±5.1	1.3±0.51	2.2±0.14	4.3±0.64
F10/2009	22	33/11	3	12.3±3.82	0.8±0.52	4.5±0.87	0.5±0.27	3.1±0.34	4.1±0.33
F9/2009	21	32/45	0.7	36.9±13.14	1.4±1.07	8.0±4.16	0.9±0.58	2.9±13.14	5.2±0.28
F6/2009	21.5	20/9	2.2	16.7±6.85	0.4±0.37	8.3±6.39	1.1±0.45	2.6±0.23	4.6±0.32
F8/2009	21	20/10	2	22.6±3.11	1.1±0.92	5.9±0.34	0.7±0.22	1.5±0.13	3.7±0.17
Mean (N=26)	21.3	30.6/17.3	2.5±0.35	23.6±0.99	0.9±0.02	8.5±1.55	0.9±0.15	3.0±0.45	4.7±0.21

F, female; M, male; T_a , ambient temperature.Means are presented \pm s.d.

Table S2. Hemolymph pressure in the mesoscutal hemocoel

Specimen ID/Date	Time (h)	T_a (°C)	Sequences		Hemolymph pressure (Pa)			Pulse amplitude (Δ Pa)	
			No./min	No. min^{-1}	Mean upper value during PPT	Mean lower value during NPT	Lowest value during NPT	PPT	NPT
F1/30.09.07	10:00	20	86/46	1.87	-41 \pm 10	-96 \pm 20	-122	3.5 \pm 0.5	\leq 1
F1/01.10.07	08:00	20	147/66	2.22	-32 \pm 2	-107 \pm 14	-230	4 \pm 1	\leq 1
M2/05.10.07	09:00	20	23/36	0.63	-50 \pm 13	-420 \pm 50	-535	10 \pm 2	2 \pm 1
M2/05.10.07	10:00	20	30/44	0.68	-32 \pm 11	-357 \pm 47	-485	8 \pm 2	\leq 1
M2/05.10.07	13:00	20	166/114	1.45	-14 \pm 6	-472 \pm 99	-774	6 \pm 2	\leq 1
M2/05.10.07	20:00	20	33/46	0.71	0 \pm 4	-405 \pm 60	-590	6 \pm 2	6 \pm 2
F3/06.10.07	16:00	21	149/51	2.92	-1 \pm 2	-78 \pm 33	-120	28 \pm 10	10 \pm 6
F3/07.10.07	06:00	21	192/155	1.24	-40 \pm 12	-268 \pm 37	-425	42 \pm 3	11 \pm 4
F3/07.10.07	21:00	21	55/28	1.96	-46 \pm 30	-286 \pm 60	-500	60 \pm 15	21 \pm 5
M4/08.10.07	08:00	22	83/42	1.97	-38 \pm 26	-378 \pm 25	-458	21 \pm 8	3 \pm 0.5
M4/09.10.07	10:00	23	34/37	0.92	-41 \pm 27	-461 \pm 40	-550	7 \pm 0.5	5 \pm 1
F5/09.10.07	23:00	23	60/34	1.76	-8 \pm 4	-48 \pm 20	-54	5 \pm 1	\leq 1
F5/10.10.07	22:00	23	45/42	1.07	-25 \pm 7	-205 \pm 37	-255	8 \pm 2	\leq 1
M9/25.11.08	08:00	20	37/20	1.85	0 \pm 1	-100 \pm 26	-130	17 \pm 3	13 \pm 2
M9/25.11.08	20:00	22	87/66	1.31	-27 \pm 9	-231 \pm 42	-380	18 \pm 6	4 \pm 1
F10/26.11.08	00:00	20	50/26	1.92	-39 \pm 13	-219 \pm 47	-249	19 \pm 4	19 \pm 11*
F10/26.11.08	17:00	20	58/43	1.3	-21 \pm 5	-255 \pm 137	-750	7 \pm 2	2 \pm 1
F10/27.11.08	01:00	21	84/36	2.33	-45 \pm 30	-257 \pm 60	-365	8 \pm 3	\leq 1
M21/02.06.09	12:00	22	148/60	2.46	-20 \pm 10	-278 \pm 48	-318	5 \pm 2	1 \pm 0.5
F23/12.06.09	17:00	23	170/56	3.03	-20 \pm 4	-86 \pm 27	-323	12 \pm 10	22 \pm 8*
F26/18.06.09	21:00	22	81/30	2.7	-29 \pm 8	-78 \pm 8	-160	4 \pm 1	1 \pm 0.5
Mean ($N=10$ flies)		20–23		1.54 \pm 0.71	-27 \pm 15	-242 \pm 136	-370 \pm 204	14 \pm 14	4.6 \pm 5.3

NPT, negative pressure pulse period in the thoracic hemocoel corresponding to the backward pulse periods of the heart; PPT, positive pressure pulse period in the thoracic hemocoel corresponding to the forward pulse periods of the heart; T_a , ambient temperature.

*High amplitudes of NPT in relation to PPT, unlike in the other data, resulting from the application of saline. These data were not considered in means and statistics.

Table S3. Intratracheal pressure in the mesoscutellar air sac

Specimen ID/Date	Time (h)	T_a (°C)	Sequences		Air pressure (Pa)	
			No./min	No. min ⁻¹	Mean upper value during PPT	Mean lower value during NPT
F2/23.11.02	06:00	21	36/32	1.12	9.2±4.3	-8.3±6
F5/03.09.08	12:00	22	56/31	1.80	10.5±5.2	-4.9±1.5
F6/03.09.08	09:00	22	34/33	1.03	13.8±1.3	-9.4±2.3
F7/03.09.08	21:00	22	41/13	3.15	10.5±2.6	-4.4±2.8
M2/28.01.09	20:00	22	129/44	2.93	1.3±0.1	-0.45±0.05
M3/02.02.09	21:00	22	77/49	1.57	0.28±0.02	-0.12±0.03
M4/05.02.09	02:00	22	67/131	0.51	0.18±0.02	-0.1±0.02
F5/06.02.09	03:00	22	34/20	1.7	0.42±0.03	-0.25±0.05
F5/07.02.09	12:00	21	31/11	2.8	0.45±0.05	-0.2±0.04
F6/18.2.09	14:00	21	114/58	1.96	0.35±0.05	-0.2±0.1
F6/19.2.09	10:00	22	32/35	0.91	0.26±0.03	-0.1±0.1
F8/20.02.09	00:00	20	49/32	1.53	0.2±0.02	-0.1±0.05
F8/21.02.09	04:00	20	67/130	0.51	0.3±0.05	-0.2±0.03
F9/22.09.09	20:00	21	10/8	1.25	19±3	-14±2
F10/01.03.09	23:00	21	27/12	2.25	0.45±0.03	-0.55±0.01
F10/03.03.09	12:00	22	129/44	2.93	0.23±0.02	-0.19±0.01
F11/08.03.09	00:00	20	141/91	1.54	57±16	-85±26
F11/09.03.09	19:00	21	41/26	1.89	1±0.05	-0.5±0.02
F12/12.03.09	08:00	21	175/55	3.1	0.10±0.06	-0.05±0.04
F13/13.03.09	22:00	20	26/22	1.18	27±2.8	-26±2.2
F13/14.03.09	20:00	20	25/20	1.25	27±2	-12±2
F13/16.03.09	02:00	21	65/18	3.61	1.1±0.3	-0.6±0.2
F14/20.03.09	12:00	20	119/33	3.6	14±2	-12±4
F14/21.03.09	07:00	21	27/11	2.45	20±2	-17±2
F14/20.3.09	12:00	22	50/31	1.6	1±0.5	-2.2±0.2
M15/24.03.09	13:00	21	43/15	2.8	13±2	-14±4
Mean (N=17 flies)		21		1.9±0.9	8.8±13	-8.1±17

NPT, negative pressure pulse period in the thoracic hemocoel corresponding to the backward pulse periods of the heart; PPT, positive pressure pulse period in the thoracic hemocoel corresponding to the forward pulse periods of the heart; T_a , ambient temperature.