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**Cover.** After about 100 years of *Drosophila* research, a new large pair of inflow valves (ostia) in the anterior heart chamber and associated 'venous' channels have been discovered by L.T. Wasserthal, and their central role in heartbeat reversals analyzed (pp. 3707–3719). Long-time recordings of heartbeat in intact flies were performed using a linear optosensor array for measurement of the transmitted IR light after lateral passage through the heart. This allowed detection of pulse direction, pulse speed and frequency along five sensor points. The new fifth set of inflow tracts helps clarify the connection between genetically distinct cell types and their ontogenetic fate.

# ***Drosophila* flies combine periodic heartbeat reversal with a circulation in the anterior body mediated by a newly discovered anterior pair of ostial valves and ‘venous’ channels**

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## Summary

Heartbeat activity in tethered adult drosophilids was recorded using a linear optosensor chip and an IR-light beam. Recording from two to five sensor elements within 250 µm along the anterior heart, it was possible to analyze periodic reversals. In intact *Drosophila melanogaster* and *D. hydei*, longer anterograde pulse periods with lower pulse rates generally alternated with shorter retrograde pulse periods having higher pulse rates. These differences are dependent on heart anatomy: a newly discovered first pair of ostia is connected to bilateral thoraco-abdominal hemolymph channels. These channels are part of a venous space separated from the abdominal hemocoel by a septum, consisting of a metanotal ridge and the pericardial diaphragm lined by a special form of fat body. The channels are sealed, and their lumen is possibly controlled by the metathoracic tergo-pleural muscle. During retrograde pulses, the heart chamber works like a suction pump, aspirating hemolymph through the first ostia from the venous channels and discharging it through a newly

described caudal opening. During forward beating, the anterior chamber receives hemolymph *via* all inflow ostia from the entire heart and drives it like a pressure pump through the narrow aorta. Also, during forward pulses, a lateral circulation occurs in the thorax as a result of the venous supply. Inhibition of abdominal mobility leads to an irregular heart rate, with pulse-wise alternating heartbeat reversals. The possible involvement of slow abdominal movements in heartbeat periodicity is discussed. The heartbeat periods are superimposed with intermittent bouts of abdominal pumping movements.

Supplementary material available online at  
<http://jeb.biologists.org/cgi/content/full/210/21/3707/DC1>

Key words: anatomy, cardiogenesis, dorsal vessel, *Drosophila melanogaster*, *Drosophila hydei*, fruitfly, heart development, inflow tract, insect heart, linear optosensor array, ostium, optocardiography, svp-lacZ.

## Introduction

Several investigations into developmental and neuronal aspects of the adult heart of *Drosophila* have recently been published. The heart of *Drosophila melanogaster* has meanwhile attracted attention as a model for the identification of genes causing adult human heart diseases (Wolf et al., 2006). However, even after about 100 years of *Drosophila* research, the regular function of the adult heart in this insect and its role in circulation are not understood because the morphological and physiological data are incomplete.

One characteristic of heart activity in many insects is periodic heartbeat reversal. It is present even in prepupae and pupae of Lepidoptera and Diptera and was first noted by Malpighi (Malpighi, 1669) (reviewed by Jones, 1977; Miller, 1997; Wasserthal, 1996; Wasserthal, 2003b). It has been shown to be a regular event in resting adults from several insect orders, including flies (Gerould, 1929; Queinsec and Campan, 1975; Thon and Queinsec, 1976; Thon, 1982; Jones, 1977; Wasserthal, 1975; Wasserthal, 1976; Wasserthal, 1982a; Wasserthal, 1982b; Wasserthal, 1996; Wasserthal, 2003a;

Wasserthal, 2003b; Angioy, 1988; Angioy and Pietra, 1995). In prepupae and pupae the function of periodic heartbeat reversal is unclear. It is suggested that in pupae of *Manduca sexta* heartbeat reversals selectively enhance the hemolymph flow towards both body extremities (Slama and Miller, 2001; Slama, 2003). In adult moths and butterflies, scarabaeid beetles and the blowfly, a periodic hemolymph shift between anterior and posterior body compartments occurs causing an alternating tracheal ventilation (Wasserthal, 1998; Wasserthal, 2003a). In *D. melanogaster*, however, physiological effects on heart rate have been investigated without taking heartbeat reversal into account (Zornik et al., 1999; Paternostro et al., 2001). Periodic changes in heartbeat frequency have recently been recorded by optoelectric registration (Slama and Farkás, 2005) and heartbeat reversals documented by indirect microscopic video techniques, and the regulating effect of neurotransmitters on anterior and posterior pacemakers has been demonstrated (Dulcis and Levine, 2005; Dulcis et al., 2005). There is, however, a lack of physiological measurements in intact adult *Drosophila* over longer periods of time and no comparison of the somewhat

controversial data with results from other flies. It remains an open question whether heartbeat reversals cause a periodic hemolymph shift between the anterior and posterior compartments in drosophilids, which could assist in tracheal ventilation.

A correct physiological analysis in drosophilids is difficult because of their small size and the relatively high pulse rate of the heart. Finding a method of recording the heartbeat from at least two measuring points, while avoiding damage and minimizing the stress caused by the need to restrain the insects, remains a challenge.

The anatomy of the *Drosophila* heart is generally thought to be well known, following the anatomical work of Miller (Miller, 1950; Rizki, 1978; Curtis et al., 1999; Dulcis and Levine, 2005). The focus of interest has now shifted to embryology of the heart at the level of gene activation and molecular signalling during morphogenesis (Bodmer et al., 1997; Cripps et al., 1999; Gajewski et al., 2000; Molina and Cripps, 2001; Lovato et al., 2002).

The ostial precursor cells of the heart tube are determined very early in development (Gajewski et al., 2000; Molina and Cripps, 2001). The number of inflow tracts is greater (seven pairs) than the three pairs of expressed ostia in the larva (Rizki, 1978), the three pairs in the pupa (Curtis et al., 1999) and the four pairs in the adult (Miller, 1950; Rizki, 1978). Comparison of the drosophilid heart with the heart in adult calliphorid flies, which have five pairs of inflow ostia and one pair of caudal outflow openings (Angioy et al., 1999; Wasserthal, 1999), suggests that the anterior enlarged heart chamber in the drosophilid heart should also have two pairs of valves, instead of only one pair as hitherto described (Miller, 1950; Rizki, 1978; Curtis et al., 1999). Furthermore it is postulated that there is an excurrent opening at the caudal end of the heart, based on the assumption that the heart transports hemolymph effectively during retrograde pulses. In order to elucidate the fly's late morphogenetic development with respect to these findings it is essential to have accurate knowledge of the adult anatomy. Therefore, preparations of the complete heart and the caudal end of *Drosophila* were analyzed using the light microscope and scanning microscope. The focus lies on the anterior heart chamber, which was analyzed in serial semithin

sections and scanning electron microscope (SEM) preparations.

## Materials and methods

### Animals

Wild specimens of *Drosophila melanogaster* Meigen were captured on rotten fruits. As they appeared very nervous and difficult to handle, the much calmer and slightly larger *Drosophila hydei* (courtesy of M. Göpfert, Cologne) were also investigated. Both insects were reared on artificial diet.

### Recording of heartbeat by a modified linear optosensor chip

Insects are partly transparent for IR-radiation, and IR light or shorter wavelength light in the visible spectrum have been used repeatedly to record heart pulses in other insects or developmental instars (see Discussion). The pulse signal obtained by IR-transmittance measurements using two sensor diodes has been validated in detail in butterfly pupae, combining this technique with contact-thermography and ultrasonic doppler velocimetry (Hetz et al., 1999). The shape of the IR-transmittance curves of a high-resolution single pulse showed that during systole of the heartbeat the condensed tissues are less transparent, reducing light transmission, and that the extension of the heart during diastole and the presystolic wave increases the transmission as the amounts of more transparent hemolymph increase. At least two measuring points are necessary to determine the direction of peristaltic contractions. However, the dimensions of the IR-sensitive diodes are much too large to be able to use more than one diode near the abdomen of the small *Drosophila*. To solve the problem, a linear multichannel sensor chip was chosen (Fig. 1A). A light beam from an IR-emitting diode (TSHA 6203, 940 nm; Conrad Electronics, www.conrad.biz) was projected by a glass fibre (230  $\mu\text{m}$  diameter) onto the side of the anterior heart region (Fig. 2). On the opposite side of this heart region a modified optosensor chip (TAOS: TSL 1402: 256x1 sensor array, maximum sensitivity at 800 nm; Plano, TX 75074, USA) was positioned along the first to fourth tergite by a micromanipulator. The fly was fixed to a piece of aluminium foil by clamping the wings under glass slides after CO<sub>2</sub>-narcosis (Fig. 1B). Attachment of the fly with its wings in the

upstroke position was the best precondition for avoiding damage. To prevent struggling and to obtain consistent results, flies were given a substrate to hold, a minute ball of soft plastic impregnated with the smell of the breeding box (see Movie 1, supplementary material). The flies were 3–7 days old. They were supplied with water and fruit juice. The measurements were performed in a temperature-controlled darkroom at  $20 \pm 0.7^\circ\text{C}$  and 55% relative humidity (RH). It was essential for the investigation that the flies felt as unrestrained as possible. Therefore, only flies that accepted the running ball and remained alive and active for at least one overnight period

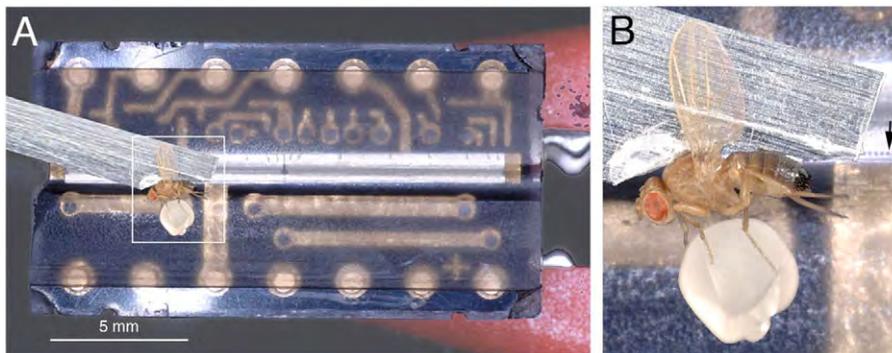


Fig. 1. Setup for recording heartbeat in *Drosophila*. (A) IR sensor-chip with adjusted holder supporting the fly. (B) *D. melanogaster* in the measuring position calmed down by holding a plastic ball. Arrow indicates the unused part of the sensor line.

were considered for data evaluation. A micromanipulator allowed precise orientation of the mounted fly between the IR emitter and sensor array until the response intensity was optimized. The signals of up to five neighbouring sensor elements, covering a surface of  $50 \times 300 \mu\text{m}$ , were processed (Fig. 2, Fig. 5). The distance between sensor elements and insect was reduced by milling and polishing a central furrow into the polymer surface above the sensor chip. A measured delay of 1 ms between consecutive elements of the linear sensor array was considered in the calculations and recording traces. The sampling rate of the 8-channel-computer interface (Powerlab: Chart 5: CB Sciences, Milford, MA 01757, USA) was 1000–40 000 Hz, depending on the number of recording channels (5, 3 or 2), respectively.

### Anatomy

Serial sectioning was done in preparations after removal of the prothorax and abdominal tip only to avoid deformation of cuticular parts in the waist, keeping the heart and surrounding tissues in a natural position. The flies were fixed in glutaraldehyde–paraformaldehyde with an initial 20 min evacuation and post-fixed in  $\text{OsO}_4$ , both in phosphate buffer. Details of fixation and embedding were as previously described (Wasserthal, 1999). Semithin sections were cut on a Reichert Ultracut 3 (Vienna, Austria) with diamond knives, stained with Toluidine Blue, analyzed in a light microscope with interference contrast (Leitz) and photographed using a Nikon D2X camera. For *in situ* preparations, the abdomen was dissected and rinsed in saline (Ephrussi and Beadle, 1936), fixed in formaldehyde and photographed in the fluid (Fig. 8A) or after embedding in purified camphene at  $45^\circ\text{C}$ , cutting with broken razor blades and sublimation of the embedding medium. The photographs were taken with a Nikon DX2 on a Leica macroscope (Fig. 11C) and a scanning EM (LEO Gemini 1530, Oberkochen, Germany).

## Results

### Analysis of heart activity

The clearest records of heart pulses were obtained using sensors arranged at the level of the anterior heart chamber beside the second to the third heart segment (Fig. 2). The latero-dorsal arrangement of the heart between beam source and sensor avoided interference of the peristaltic movements of the intestine with the heart pulses. In the waist region, however, the activities of heart and intestine were superimposed (see below and Fig. 6). The main difficulty during recording of heartbeats arose from the necessity to leave the abdomen unrestrained so that it could change its length. Under these conditions the abdomen was free to perform pumping movements, which obscured the recording of heartbeat. Vertical movements of the dorsal abdomen affected the transmittance. Fortunately, in fully resting flies these

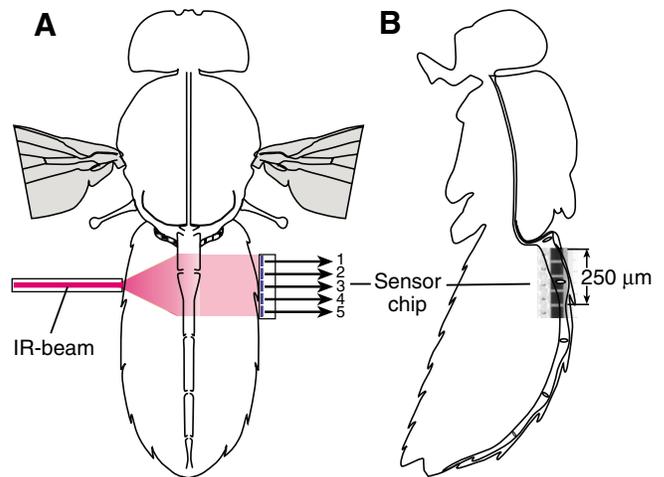


Fig. 2. Illustration demonstrating orientation of the IR-light beam and the opposite five sensor elements (1–5) for recording pulsations of the anterior heart (second segment) in *D. melanogaster*. Proportions to scale.

movements stopped intermittently and then were only performed in bouts (Fig. 3A). The duration of the bouts in *D. melanogaster* lasted from about 1 to 20 s, with a contraction rate

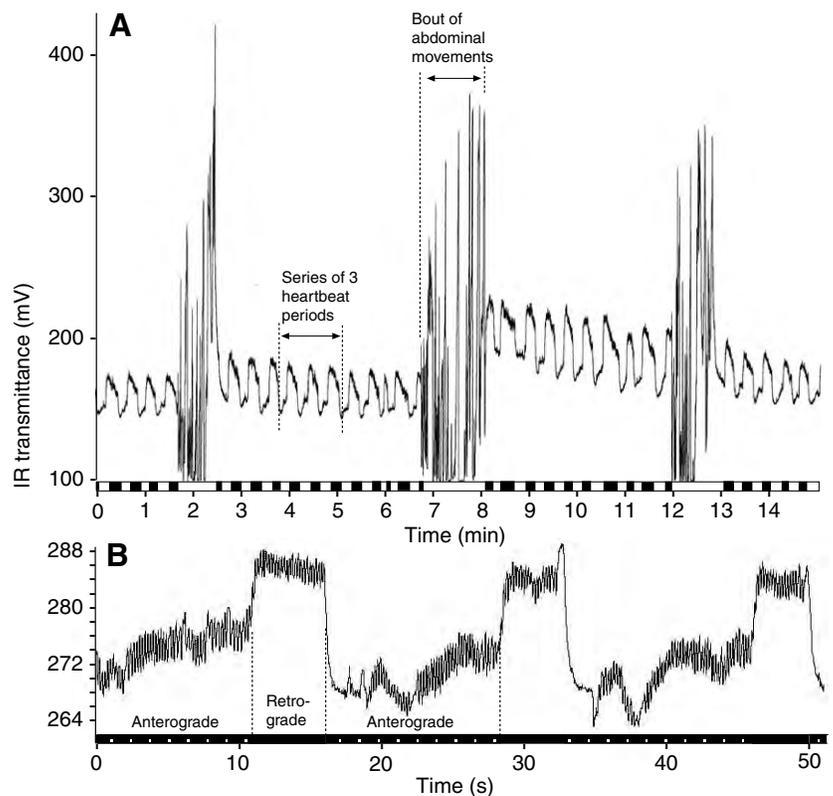


Fig. 3. Survey of heartbeat reversals, recorded by the IR-sensor beside the second abdominal segment. Black bars represent the periods of retrograde heart pulses. (A) Regular repetition of heartbeat reversals and intermittent bouts of abdominal contractions (*D. hydei*, male 3). (B) Series of three heartbeat periods in *D. melanogaster* (female 2). During retrograde pulses the mean transmittance level is higher than during forward pulses.

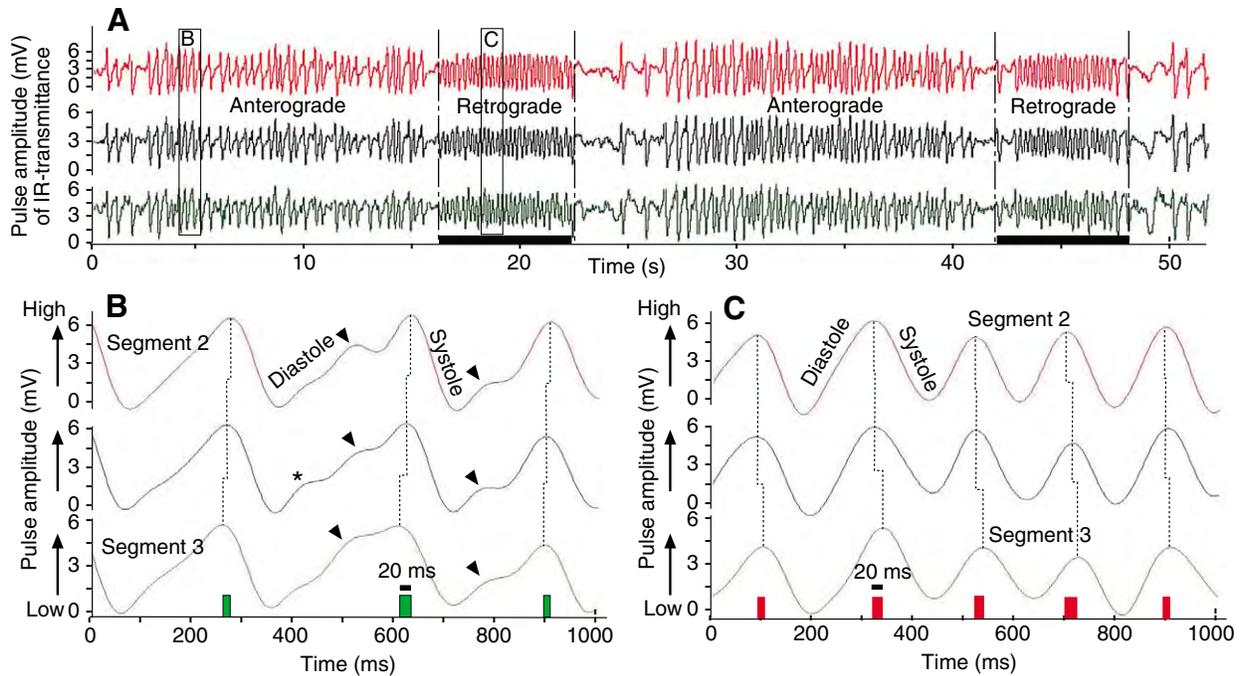


Fig. 4. Heartbeat reversals in *D. hydei* (female 6). The pulse sequences of three traces are sampled at 2000 Hz and the different mean levels of transmission are equalized by use of a high-pass filter of 0.5 Hz. (A) Survey of two series of heartbeat periods. (B,C) Shorter time periods with individual heart pulses. Detail from the boxes marked in A. The metachronous delay of the pulse wave is recorded by three sensor elements along the abdominal segment 3 (green), inter-segmentally (black) and segment 2 (red). (B) Anterograde pulses show typically a double peak (arrowheads) or even further peaks (asterisk). (C) Retrograde pulses show only one peak. The peak of highest transmission corresponds to the moment when the heart is maximally distended by the presystolic wave. To illustrate the metachrony of the pulse wave, the maximum peaks are connected (dotted lines) and the resulting intervals are projected as a bar on the  $x$ -axis. Green anterograde, red retrograde.

of 0.1–0.2 Hz, and in *D. hydei* bout duration was about 10–100 s, with a contraction rate of 0.2–0.4 Hz. Analysis of heart pulses was only possible during the interbout phases between these abdominal movements. The pulse direction was analyzed by measuring the metachrony of the pulse recorded at 5, 3 or 2 sensor points after using a high-pass filter of  $\sim 0.5$  Hz

for suppression of the periodic up- and down-changes in mean transmittance (Fig. 4).

There are no fundamental differences in the optical recordings, frequencies and period lengths between the two *Drosophila* species: the heart alternates between pumping hemolymph in a forward (=anterograde) and a reverse

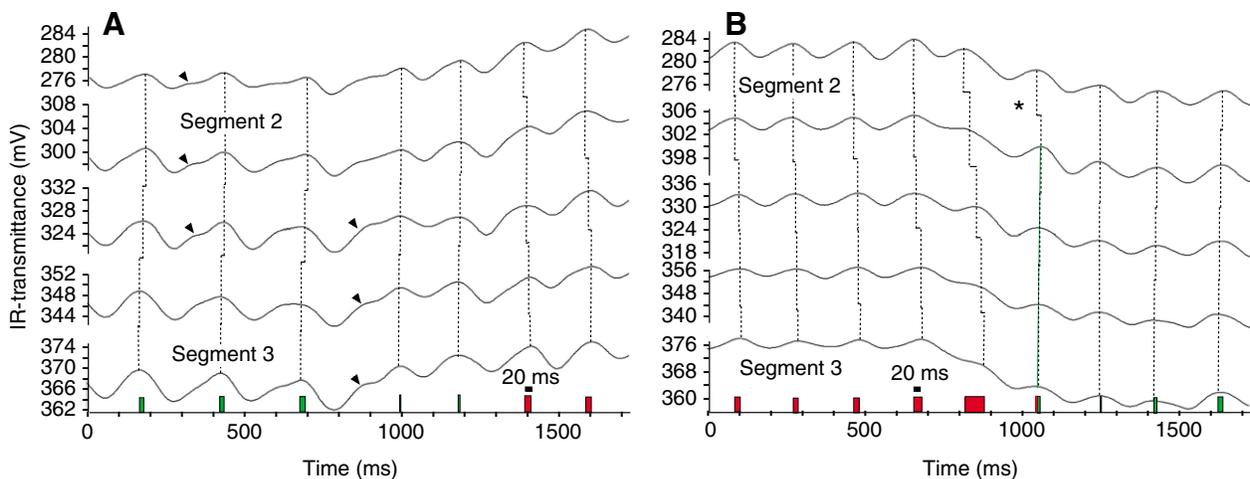


Fig. 5. Directional change of heartbeat, analyzed by the metachrony along five sensor sites in *D. melanogaster* (female 2). (A) Transition from anterograde to retrograde beating. Arrowheads indicate double peaks during diastole. (B) Transition from retrograde to anterograde beating. The first anterograde pulse collides with a retrograde pulse at the anterior sensor point (asterisk). Explanations as in Fig. 4.

Table 1. Duration of anterograde and retrograde pulse periods, pulse rate and pulse speed of the heart in *Drosophila melanogaster* and *D. hydei*

Species	Anterograde			Retrograde		
	Period duration (s)	Heart rate (Hz)	Pulse speed (mm s <sup>-1</sup> )	Period duration (s)	Heart rate (Hz)	Pulse speed (mm s <sup>-1</sup> )
<i>D. melanogaster</i>						
F1/06	16.0±3.5	3.0±0.6	2±0.5	4.1±0.7	4.3±0.4	7±3
F2/06	12.4±4.9	4.6±0.3	50±26	3.5±0.9	5.3±0.2	24±11
F3/06	17.2±7.9	5.2±0.5	9±7	6.5±2.3	6.1±0.2	6±3
M1/06	10.9±4.4	4.1±0.2	12±6	5.8±0.1	5.6±0.4	38±23
Mean	14.1±2.9	4.2±0.9		4.9±1.4	5.3±0.8	
<i>D. hydei</i>						
F6/05	7.1±1.5	2.5±0.6	6±2	4.0±1.0	5.3±0.3	19±2
M7/05	24.4±9.5	4.2±0.2	8±4	3.6±0.5	5.5±0.2	50±12
M9/05	13.8±3.5	4.2±0.3	10±5	3.1±0.3	5.1±0.2	17±7
F2/06a	21.2±7.0	3.3±2.7	23±20	4.6±0.7	4.4±0.3	20±10
F2/06b	10.0±1.7	3.8±0.3	13±8	5.2±0.7	4.9±0.2	21±6
F3/06a	6.6±3.3	3.4±0.1	14±9	11.5±2.9	4.9±0.2	50±40
F3/06b	25.7±5.2	4.0±0.2	50±35	3.3±4.91	4.9±0.1	66±40
F6/06	19.4±6.6	2.7±0.4	66±31	6.5±2.2	4.6±0.5	43±37
M3/06	41.4 ±10.5	4.0±0.1	13±1	8.8±2.9	4.6±0.2	13±11
M2/06	12.1±1.8	3.0±0.2	47±22	4.5±0.7	4.6±0.1	32±31
M4/06a	33.4±9.3	2.8±4.6	14±3	9.5±2.7	4.7±0.4	13±10
M4/06b	11.7±3.7	2.8±0.4	27±26	7.0±1.1	5.1±0.2	21±15
M4/06c	9.4±3.0	4.0±0.3	15±9	8.6±2.5	5.5±0.1	25±16
Mean	18.1±11.1	3.4±0.7		6.9±3.2	4.9±0.3	

*D. melanogaster*, N=4; *D. hydei*, N=9.

24 period sequences were evaluated per individual and day.

Notation such as F or M4/06a, b, c means that female or male 4 was measured on 3 consecutive days.

(backward=retrograde) direction with a very regular periodicity. The anterograde heartbeat periods of *D. melanogaster* are generally longer (14.1±2.9 s) than the retrograde periods

(4.9±1.4 s) (18.1±11.1 s and 6.9±3.2 s, respectively, in *D. hydei*) (Table 1 and Fig. 3B). In younger, fully hydrated flies, the retrograde pulse periods are almost as long as the anterograde periods (Fig. 6B). The anterograde pulses of *D. melanogaster* generally have a lower frequency (4.2±0.93 Hz) than the retrograde pulses (5.3±0.8 Hz); N=4 flies, with 24 periods calculated per fly (*D. hydei*: 3.4±0.7 Hz and 4.9±0.3 Hz, respectively, N=9) (Table 1). The velocity of the peristaltic wave is extremely variable and ranges from ~1.5 mm to

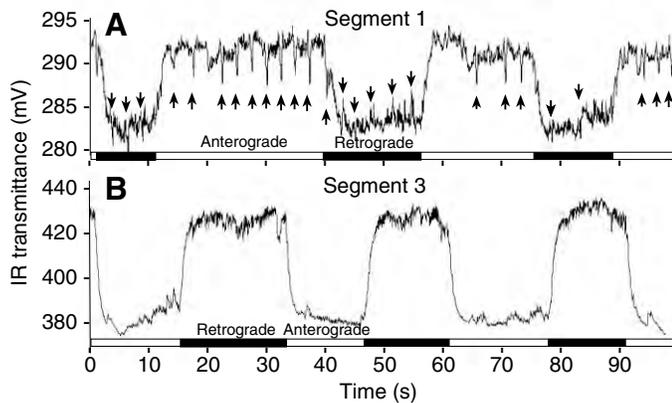


Fig. 6. Comparison of periodic heartbeat traces of first with third heart segment in *D. hydei* (male 4). (A) Sensor beside the waist at the transition of the first heart segment to the aorta. The mean IR-transmittance is decreasing during retrograde pulsations, when no hemolymph is pumped through the aorta. Pulses, probably of the intestine, with a lower frequency are superimposed on the heart pulses (arrows). (B) Same individual, with sensor beside the third heart segment the next day. Mean IR-transmittance is increasing during retrograde pulsations, when hemolymph is accumulating in the abdomen.

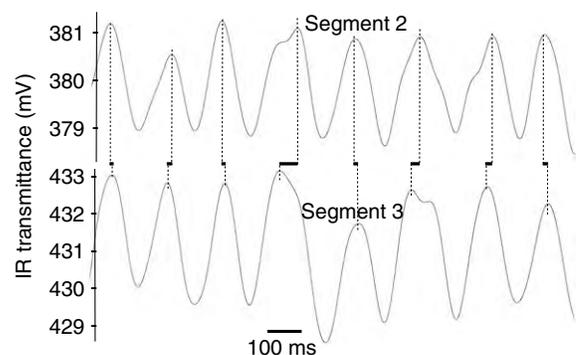


Fig. 7. Influence of restrained abdomen tip on the heart pulses in *D. hydei* (female 3). This form of pulsewise alternating heartbeat reversals can last for hours as long as the abdomen is prevented from length changes. It documents the importance of the role of abdomen for regular heartbeat periodicity.

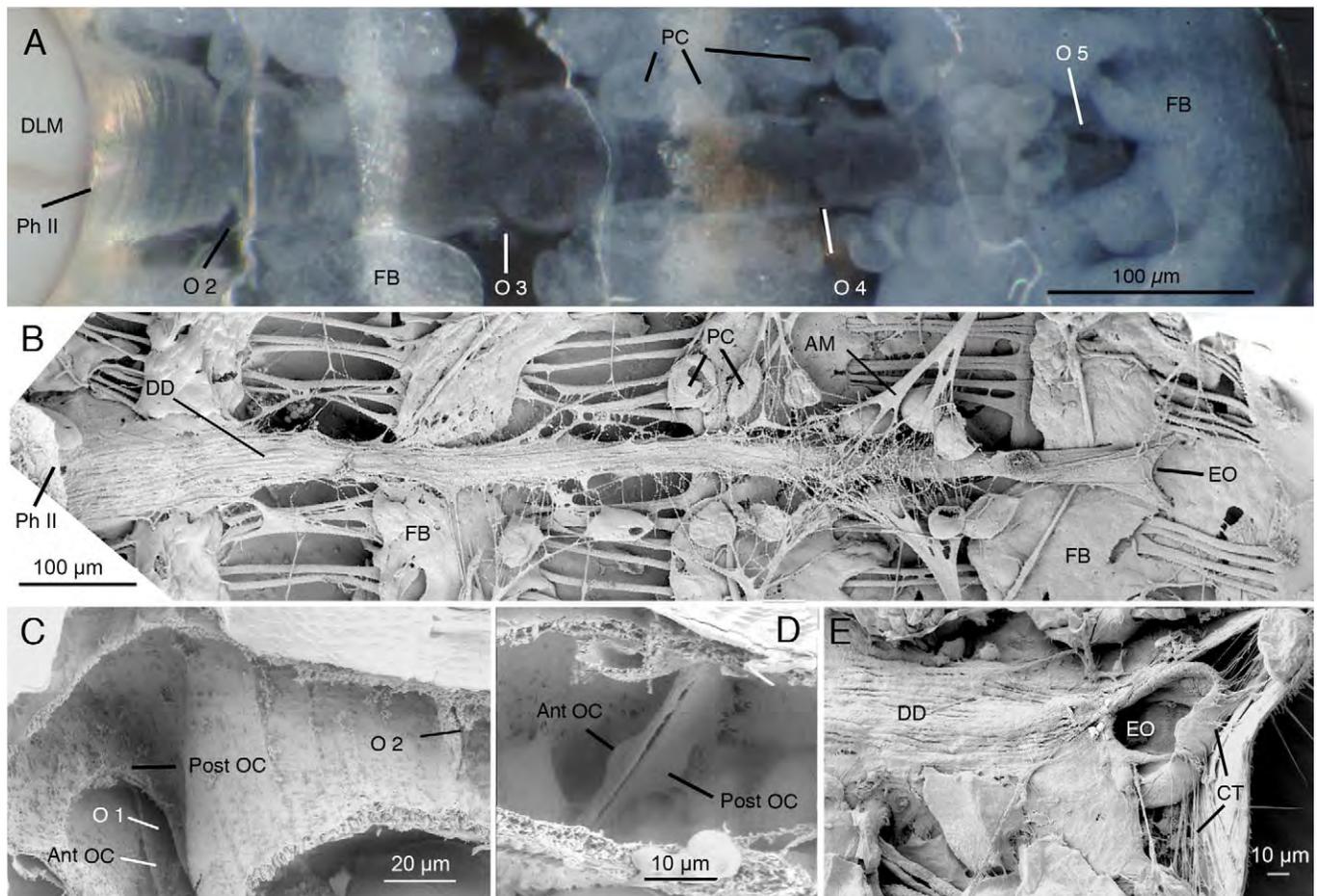


Fig. 8. Anatomy of the heart and its incident and excurrent openings in *D. melanogaster*. (A) Light micrograph of the entire heart, ventral view. Ostia 2, 3 and 4 (O2, O3, O4) are clearly visible through the transparent dorsal diaphragm. Fat body (FB) and pericardial cells (PC) cover the posterior part of the heart. DLM, dorso-longitudinal flight muscles attached to the mesophragma (Ph II). (B–E) SEMs. (B) Ventral view of the entire heart. The dorsal diaphragm (DD) with its longitudinal muscles is visible, covering the ventral surface of the heart. The excurrent opening (EO) is visible through the pair of caudal suspending muscles. AM, alary muscles. (C) Longitudinal cut of anterior heart chamber with first ostium (O1). Anterior (Ant OC) and posterior valve cells (Post OC) protrude into the heart lumen. (D) Third ostium with closed valve lips. (E) Caudal end of the heart with excurrent opening, suspending muscles severed to expose the relaxed opening and the connective tissue strands (CT).

$\sim 100 \text{ mm s}^{-1}$  in both directions. This means that a peristaltic wave needs about 5–240 ms to traverse the 1 mm long heart of *D. melanogaster*. The anterograde pulses are less regular and single pulses often look more complex by the addition of one or more peaks (Fig. 4B, Fig. 5A). These additional peaks are generally not visible at the beginning of the forward pulse periods (Fig. 5B) but become obvious in the course of the period (Fig. 5A). It is assumed that the anterior heart chamber during diastole is filled by hemolymph during the presystolic wave, which in the course of the forward pulse period flows against an increasing back-pressure and therefore becomes inhomogeneous (graduated) and reduces the pulse speed (compare Fig. 5A,B). The retrograde pulses are more regular and are characterized by a single peak (Fig. 4C, Fig. 5B). The original unfiltered recordings with the slow periodic changes in mean level of transmittance provide additional information. At the second and third segment the mean level of transmittance during retrograde pulses is generally higher than during anterograde pulse periods (Fig. 3, Fig. 6B). The transmittance

increases during the first retrograde pulses and achieves a nearly constant level, slightly undulating. At the beginning of the anterograde pulse periods the transmittance decreases rapidly during the first few pulses and remains on a lower but variable level. It is noteworthy that with mobility of the abdomen restricted by gluing the caudal end, length changes are prevented, so that the mean level of IR-transmittance remains rather constant during anterograde and retrograde pulses. Under these conditions, the heart reverses its beating direction irregularly or alternates its pulse direction repetitively with each pulse (Fig. 7). The heart seems to be unable to perform the normal sequences of periodic reversals. It is concluded that apart from the above-described intermittent bouts of abdominal movements, which are independent from the heartbeat periodicity, the abdomen changes its length and volume in parallel with the heartbeat reversals. The increase of mean transmittance during retrograde pulses is a consequence of the augmenting hemolymph volume combined with abdominal expansion. The rapid decrease of mean transmittance during

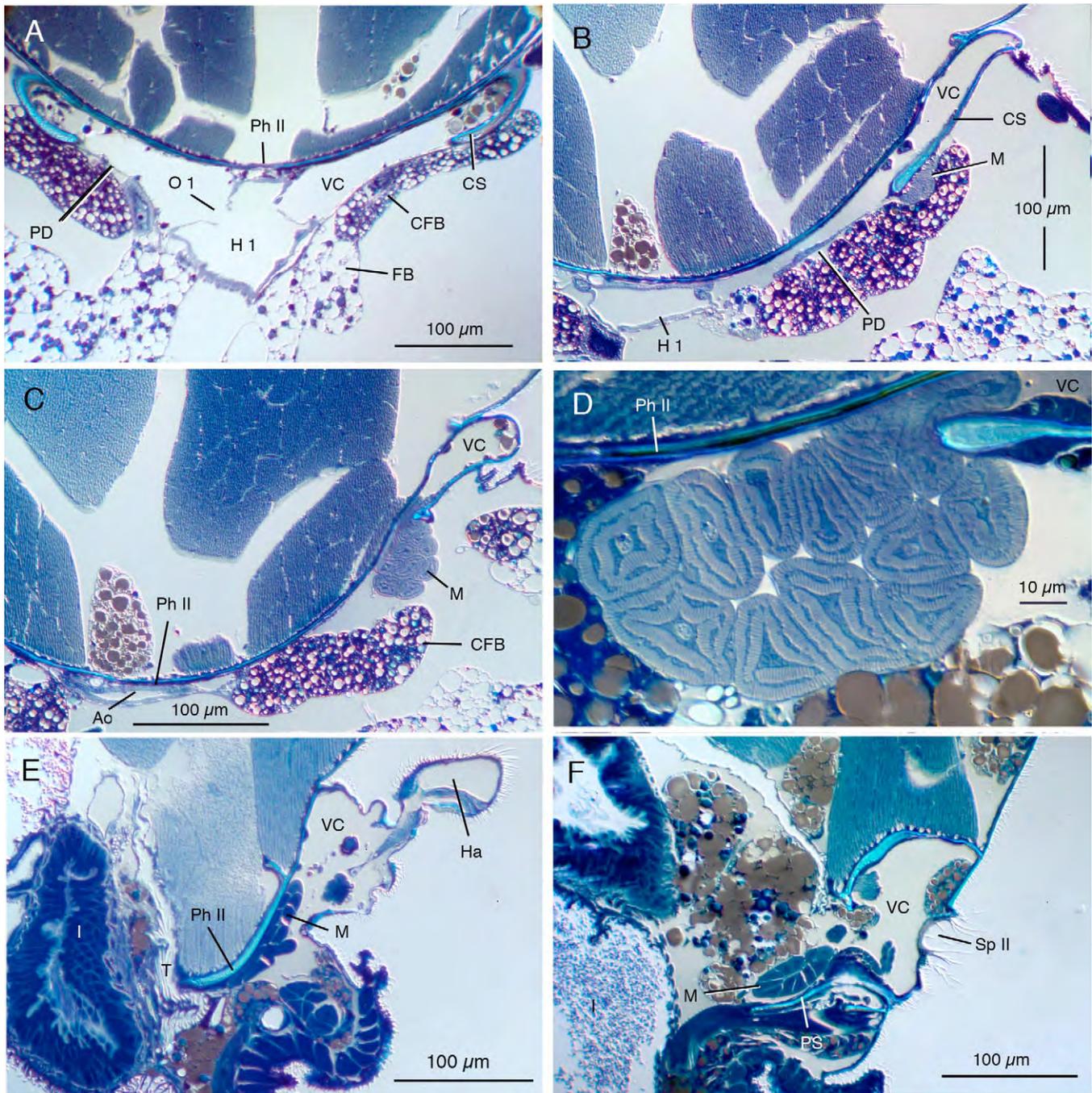


Fig. 9. Horizontal semithin sections of the first abdominal segment/posterior thorax in *D. hydei*. (A) Section just below the first abdominal tergite, showing the heart chamber (H1) with first ostia (O1) and venous channel (VC) bordered by a cuticular septum (CS), pericardial diaphragm (PD) and constitutive fat tissue (CFB). (B) Section 32  $\mu\text{m}$  below A, showing the venous channel and the tergo-pleural muscle (M) attached to the cuticular septum. (C) Section 30  $\mu\text{m}$  below B. The aorta (Ao) is collapsed. The venous hemolymph channel is bordered by the tergo-pleural muscle. (D) Tubular tergo-pleural muscle. Detail similar to C. (E) Section 180  $\mu\text{m}$  below B at the basis of the haltere (Ha). (F) Section 268  $\mu\text{m}$  below B. Venous channel above the metathoracic spiracle (Sp II). The tergo-pleural muscle (M) is close near the pleural septum (PS), where it is attached 98  $\mu\text{m}$  more ventrally. FB, fat body; I, intestine; Ph II, mesophragma; T, trachea.

anterograde pulses is caused by abdominal contraction under reduction of hemolymph volume in the abdomen and the denser arrangement of the tissues.

In the curves recorded from the first abdominal segment at the transition from heart chamber to aorta, the mean transmittance

is lower and the anterograde pulses have a higher transmittance level (Fig. 6A), supposedly reflecting the hemolymph wave distending the anterior heart chamber and aorta. By contrast, during retrograde pulses the transmission decreases, due to the fact that no hemolymph passes through the aorta.

Here in the waist, the abdominal movements and volume changes have only little effect due to the dense arrangement of organs and fat body. However, another type of pulse with greater amplitude and low frequency of 0.3–0.4 Hz interferes with the heart pulses (Fig. 6A, arrows). This interference is obvious during the anterograde pulse periods, but almost hidden during the retrograde pulses. In the waist, the vertical part of the anterior heart and the aorta directly adjoin the intestine. It is inevitable that in this anterior, narrow part of the abdomen the peristalsis of the intestine influences the IR light beam. These peristaltic movements occur continuously and should not be confused with the intermittent abdominal pumping movements.

#### Anatomy of heart and associated structures

As the postulated ostia were suspected to be in the anterior part of the heart, which is curved ventrally, the waist region was sectioned horizontally to obtain cross sections of this part at its site of contact with the adjacent mesophragma. A pair of ostial valves was found in the anterior part of the first heart chamber immediately behind the mesophragma, just above the transition

to the aorta (Fig. 8C, Fig. 9A, Fig. 10B, Fig. 11B). Here the frontal surface of the first heart chamber is directly attached to the mesophragma by threads of connective tissue (Fig. 10E). The ostial valve lips are oriented into the heart lumen (Fig. 10B). The anterior lips arise directly from the attached frontal heart margin. This position is the reason why, in most preparations, the heart is rupturing along the anterior ostial slits and why the first ostia were overlooked in all previous analyses. SEM micrographs of the longitudinally split anterior heart chamber show the first ostial lips, which are tightly closed and deeply protrude into the heart lumen in such a way that they allow an inflow only during diastole and are closed by the action of being pressed against the anterior wall of the mesophragma during systole (Fig. 10B). The second pair of ostia, which until now was thought to be the first one, is smaller and about 100–300  $\mu\text{m}$  behind the ‘new’ first pair, depending on the overall size of the fly (Fig. 8C). In contrast to the first ostia, all posterior ostial pairs (numbers 2–5) are visible as deep inflections of the inwardly projecting lips in the liquid preparations (Fig. 8A).

The relaxed anterior heart chamber has a wide lumen and is

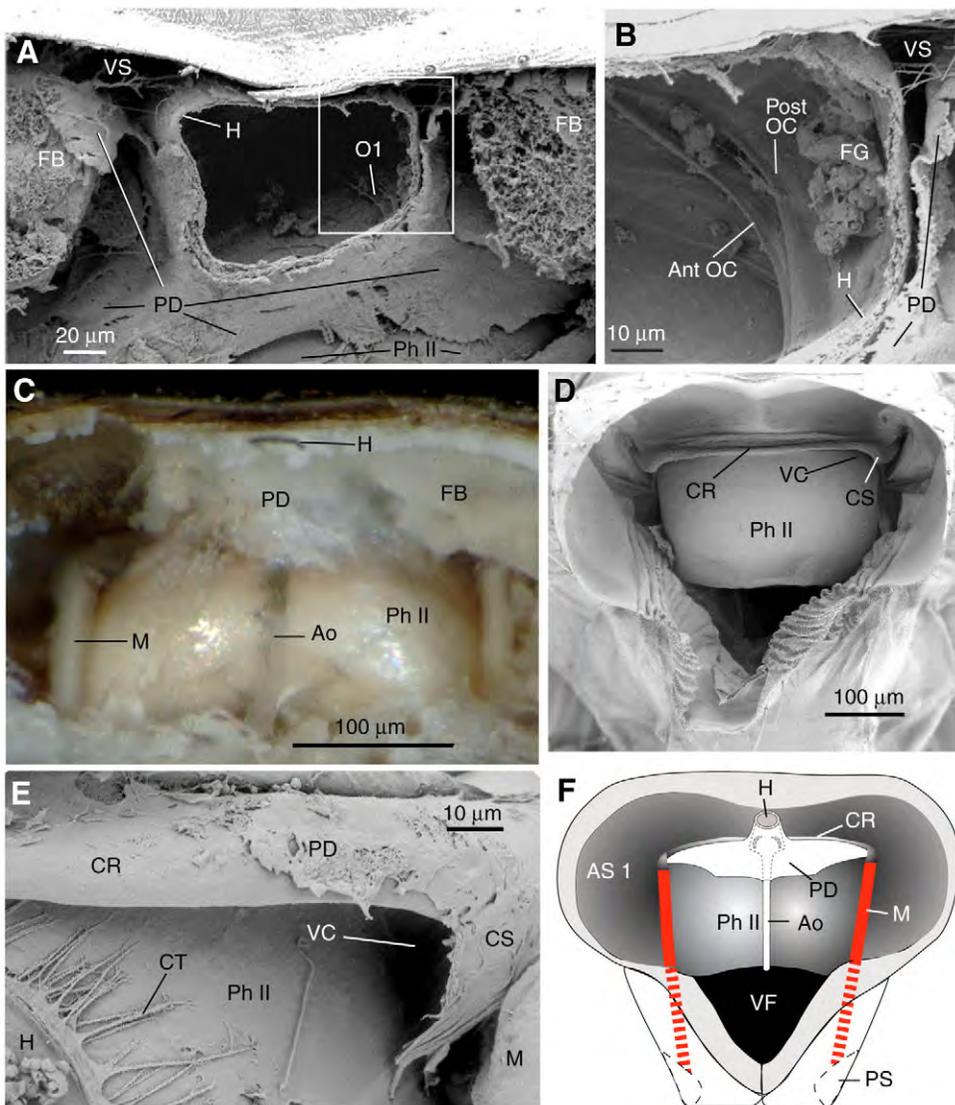


Fig. 10. The venous space (VS) and its connection to the anterior heart (H) in the first abdominal segment in *D. melanogaster*. View towards the mesophragma (Ph II). (A,B,D,E) SEMs; (C) light microscopy. (A) Opened anterior heart chamber, surrounded by the pericardial diaphragm (PD) and the fat body (FB). (B) Detail from A, showing the protruding valve cells of the first ostium. (C) Light micrograph of the first abdominal segment, showing the tergo-pleural muscles (M), pericardial diaphragm (PD) and aorta (Ao). Sectioned at the level of the posterior end of the (collapsed) conical heart chamber (H). Most fat body (FB) on the left side is removed. (D) NaOH-maceration preparation of the anterior abdomen. Cuticular ridge (CR), arising from the posterior metanotum and giving rise to the cuticular septum (CS), bordering the venous channel. (E) View as in D after removal of the pericardial diaphragm to show the anterior heart attachment by connective tissue strands (CT) and opening of the venous channel. PD, tissue remnants of the pericardial diaphragm. (F) Schematic illustration of components involved in hemolymph transport of the anterior heart. Ant OC, anterior ostial valve cell; AS 1, first abdominal segment; FG, fat granule; M, tergo-pleural muscle; O1, first ostium; Post OC, posterior valve cell; PS, pleural septum; VC, venous channel; VF, ventral foramen; VS, venous space.

suspended inside the muscular pericardial diaphragm by connective tissue strands. The large 'conical' heart chamber in the first and second abdominal segments tapers towards the slender caudal end (Fig. 8A). The caudal heart tube ends with a funnel-like opening. It is suspended by a pair of diverging muscles fixed at the posterior margin of the sixth abdominal segment (Fig. 8B) and dorsally by connective tissue strands (Fig. 8E). Towards the thorax, the transition of the heart chamber into the thin-walled narrow aorta is abrupt. In contrast to the heart, the aorta is not surrounded or suspended by muscles or connective tissue strands. It has a narrow lumen, which in the relaxed state is dorso-ventrally flattened (Fig. 9C). The aorta has no inflow openings and terminates with an outflow opening in the posterior head (Miller, 1950).

At the ventral face, the heart is connected to and covered by the dorsal diaphragm along its whole length (Fig. 8B). In the first abdominal segment, the dorsal diaphragm fully surrounds the anterior part of the conical heart chamber and forms a dense muscular pericardial diaphragm. It is the inner layer of the pericardial septum around the 'venous' space (Fig. 9A,B, Fig. 10). Along the rest of the heart, the dorsal diaphragm is tightly attached to the ventral heart and leaves the lateral heart with the ostia uncovered and accessible for the abdominal hemolymph. Its muscles are oriented longitudinally and may shorten the heart tube when contracted. There are weak alary muscles in the third and fourth abdominal segments but two pairs of strong fans of alary muscles suspend the heart in the fifth abdominal segment. A pair of caudal suspending muscles arise directly from the heart tube.

The new first pair of ostia has a central function because it is exclusively connected to the thoracic hemocoel *via* a pair of hitherto overlooked lateral hemolymph channels. These channels are a part of the greatly modified posterior metathoracic hemocoel. The dipteran thorax is characterized by the displacement of the mesothorax into the metathorax due to the mighty dorso-longitudinal flight muscles (Ferris, 1950). The mesophragma medially intrudes into the first abdominal segment and serves as attachment site of the anterior heart wall and the ventral part of the pericardial septum (Fig. 10, Fig. 11B). The metanotum is reduced to a cuticular dorsal ridge extending laterally as a cuticular septum, which borders the lateral channel. Ridges and septum serve as attachment site for the lateral and dorsal part of the pericardial septum (Fig. 10D,E).

The pericardial septum is composed of the pericardial diaphragm and an outer layer of dense 'constitutive' fat body (Fig. 9A,B, Fig. 10A,C). It shields the venous space against the remaining abdominal hemocoel. As all other ostia, including the second pair, lie outside the venous space, only the first pair of ostia receives hemolymph from the thorax. Thus, in *Drosophila* a 'venous supply' to the anterior heart is established by the lateral channels.

The channels continue between mesophragma and lateral metathorax along the base of the haltere (Fig. 9E) and communicate with the hemocoel behind the metathoracic spiracles (Fig. 9F). At the level of the aorta, the lateral channels are no longer bordered by the pericardial septum but by a tubular muscle (Fig. 9B–D, Fig. 10C,F). This muscle is the tergo-pleural muscle of the metathorax. It is partly identical with the

upper part of the tergo-sternal depressor, No. 86, described by Miller (Miller, 1950). It originates from the outer dorsal free edge of the cuticular septum (Fig. 9B) and descends anterior-ventrally. This muscle is stretched over the convex-curved mesophragma before it enters the ventral foramen of the thorax (Fig. 10C,F). The ventral end is fixed at the stiff pleural apophysis arising from the suture between the pre-episternum 3 and epimeron 3 (Fig. 9F, Fig. 10F). The function of this muscle remains unclear. As it is attached to the channel septum of the metanotum, which is elastically connected to the mesonotum, it may either approach the septum towards the mesophragma and reduce the lumen of the channel or remove it and dilate the channel lumen. This muscle could change the flow volume of the venous channel in coordination with the heartbeat reversals.

In flies no ventral hemocoelic connection between thorax and abdomen (e.g. a perineural sinus) exists, due to the lack of an abdominal ventral nerve cord and a ventral diaphragm (Richards, 1963). The ventral foramen in the waist between thorax and abdomen (Fig. 10D,F) is filled and probably tightened by the intestine with its appendages and sealed by muscles and fat body (Fig. 9E,F, Fig. 10C). Thus no other than the lateral hemocoel connection between thorax and abdomen is known. The separation of the thoracic hemocoel from the abdominal hemocoel divides the body into two compartments as a precondition for a periodic hemolymph shift by heartbeat reversal.

## Discussion

### *Different methods of heartbeat analysis lead to different interpretations of direction of pulse wave*

A characteristic of the heart in larger flies is the periodic heartbeat reversal (Gerould, 1929; Brazeau and Campan, 1970; Queinnec and Campan, 1975; Jones, 1977; Wasserthal, 1982b; Angioy and Pietra, 1995). Attribution of pulse rate to direction of peristaltic wave is, however, controversial. In earlier investigations on the heart of the adult *Calliphora* and *Protophormia*, the pulses with the lower pulse rate were interpreted to be retrograde (Brazeau and Campan, 1970; Queinnec and Campan, 1975; Thon and Queinnec, 1976; Angioy and Pietra, 1995). However, with contact thermographic studies using local heat-marking in intact *Calliphora*, the pulses with higher frequency are shown to run retrograde (Wasserthal, 1982b; Wasserthal, 1999; Wasserthal, 2003a). The methodological dilemma due to the small size in *Drosophila* is now solved by use of the IR-radiation+linear optosensor array method introduced in this paper, which allowed measurement of the heartbeat in tethered, intact flies over a long period (up to several days) and determination of the pulse speed and direction.

Photosensors have been used to record insect heartbeat [*Bombyx*, *Xylotrupes* (Tachibana and Nagashima, 1957); *Schistocerca* (Phaneuf et al., 1973); *Sarcophaga* (Williams et al., 1968); *Schistocerca*, *Sarcophaga* (Lhotsky et al., 1975); *Ornithoptera* pupae (Hetz et al., 1999); *Manduca* pupae (Slama, 2003)]. In *Drosophila* pupae and a few adults, Slama and Farkas used a single IR photodiode for heartbeat recordings under transmitting and reflecting light (Slama and Farkás, 2005). But the pulse direction could not be recorded using a single sensor element and thus they attributed the higher frequency pulses to

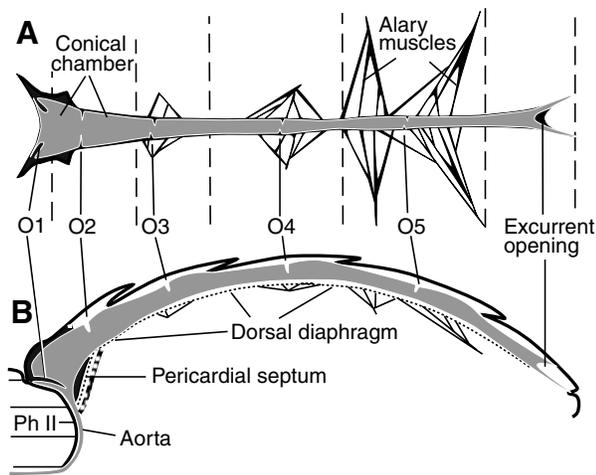


Fig. 11. Schematic drawing of the heart with its ostia and the dorsal diaphragm in *Drosophila*. (A) Dorsal view, (B) lateral view. O1–O5, incurrent ostia of abdominal segments 1–5; Ph II, mesophragma.

the anterograde direction on the basis of visual observation. Dulcis and Levine processed heart videos electronically and transformed light density fluctuations into curves of heart pulses (Dulcis and Levine, 2005). They analyzed up to three points along the heart in order to detect the pulse direction. They also attributed the higher frequency pulses to the anterograde direction and correlated anterograde and retrograde beating with phasic volumetric changes of the conical heart chamber. They called the retrograde beating phase with higher volume ‘diastolic’ and the anterograde beating phase with smaller volume ‘systolic’. As the heart continues to pulsate during these phases, the terms ‘systole and diastole’ and ‘mini systole and diastole’ are somewhat misleading and the force or mechanism for the slow volume changes of the conical chamber (or is it the pericardial diaphragm? see below) remains unclear.

In the IR-multisensor recordings in intact *Drosophila* (wings and legs intact, abdomen free to move), the phases with higher pulse rate are generally retrograde and these pulses are more regular than the slower anterograde pulses, which are characterized by double or even more complex peaks during diastole.

The latter diagnosis is confirmed by x-ray videos from the heart of *Calliphora* performed at the ESRF-synchrotron facility, Grenoble. These clearly show that the higher frequent pulses coincide with retrograde pulsations (Wasserthal et al., 2006). The contradictory results may be explained by the different experimental procedures. If the wings and legs are severed and the hemolymph and tracheal spaces are opened, as in the experiments of Dulcis and Levine (Dulcis and Levine, 2005), the conditions of pressure and hemolymph supply from the lateral thorax are possibly modified and may influence the diastolic volume.

*The newly discovered first ostium and caudal opening of the heart in Drosophila have important functions in hemolymph transport*

Nearly all recent papers on the *Drosophila* heart focus on regulation and innervation, with no special attention given to the

functional design and proportions of the heart tube, which completely differs from other – even holometabolous – insect orders such as Lepidoptera and Coleoptera (Gerould, 1929; Jones, 1977; Wasserthal, 1996; Wasserthal, 2003a). In order to understand the mechanism of heartbeat reversal it is essential to know the number and position of heart openings. In adult *Drosophila* heart, all authors have described only four pairs of inflow ostia and a closed caudal end (Miller, 1950; Rizki, 1978; Curtis et al., 1999). In fact just the newly discovered first pair of ostia reported here and the caudal opening are necessary for retrograde hemolymph transport to function. The morphology of the enlarged anterior heart chamber, with two pairs of ostia, is homologous to that of calliphorid flies (Angioy et al., 1999; Wasserthal, 1999). The caudal excurrent opening in *Drosophila* is, however, unpaired, in contrast to the calliphorid flies with paired openings.

The difference in pulse rates of retrograde pulses with the higher frequency in flies but with lower frequency in Lepidoptera reflects their different anatomy with different refilling and outflow conditions. The lepidopteran heart also has ostia in the thoracic aorta and two-way ostia in the abdomen with no caudal opening (Gerould, 1929; Jones, 1977; Wasserthal, 2003b). Diastolic filling during retrograde pulses of the heart occurs in a complex way by action of the tergal pulsatile organs *via* these aortal ostia, and the hemolymph must be pressed through the posterior ostia (Wasserthal, 1996). In flies, during retrograde pulses, the anterior heart chamber with the first large ostia receives hemolymph directly from the lateral thorax and discharges it through the caudal opening. Thus, the retrograde outflow in flies encounters less resistance than in moths and butterflies and this may be the reason why the backward pulses have a relatively higher frequency in flies than the forward pulses, similar to scarabaeid beetles, which also have an open caudal heart end (Wasserthal, 1996).

*The mechanism of thoraco-abdominal hemolymph flow by ‘venous’ channels*

As a special trait in *Drosophila*, the first heart ostia are found to be connected to thoraco-abdominal channels. They provide a flow passage from the lateral thorax along the bases of the halteres and further to the dorsal abdomen, directly to the pericardial space around the anterior heart chamber. The pericardial septum shields the pericardial space and the hemocoel of the lateral channels against the rest of the abdominal hemocoel and thus functions in a similar way to the anterior wall of the large abdominal air sacs in *Calliphora* (Wasserthal, 1999). The venous channels are crucial for understanding hemolymph transport from thorax to abdomen during backward beating. Retrograde hemolymph transport *via* the aorta has been considered by some authors (Angioy and Pietra, 1995; Angioy et al., 1999). Dulcis and Levine note that ‘the conical chamber lumen rapidly increased to reach the maximum diastolic volume soon as the retrograde (!) beat started’ (Dulcis and Levine, 2005), leaving the question of inflow undiscussed. Effective aortal retrograde pulses are, however, ruled out by structural constraints: the aorta is dorso-ventrally flattened when relaxed. It is thin-walled and contains no striated muscles (Miller, 1950). Without a suspending muscle or connective tissue layer it cannot actively aspire

hemolymph but must collapse when the diastolic heart sucks hemolymph during retrograde pulsations. Thus functional preconditions for retrograde heart pulses in *Drosophila* differ fundamentally from those of most other insect orders with heartbeat reversals, which possess inflow ostia in the thoracic aorta (see above). Preconditions for diastolic filling of the heart are essential for any heart functioning (Vogel, 1992). With its thoraco-abdominal channels connected to its anterior pair of ostia, the *Drosophila* circulatory system evolved a venous pathway for effective retrograde transport from thorax to abdomen. This compensates for a number of fundamental reductions compared to a phylogenetically older plan, such as aortal ostia and the ventral diaphragm for retrograde hemolymph transport.

*The alternating function of the anterior heart chamber as a pressure pump and suction pump*

My results lead to the following conclusions. The diastolic filling process of the anterior chamber during forward and backward pulses is fundamentally different. During anterograde pulsations, the heart chamber receives hemolymph from all ostia and gets dilated by the presystolic wave (presystolic notch) (Yeager, 1939), represented by the graduated peak of the anterograde pulses. The complete volume of the anterior chamber is then pressed through the narrow aorta. The need to overcome the internal resistance of the aorta is possibly the

reason for the lower pulse rate and the graduated pulse peaks. During retrograde pulses, the hemolymph is sucked by the anterior heart chamber directly through the first pair of large ostia from the lateral thorax *via* the venous channels and is discharged through the caudal opening. While the first ostia are essential for hemolymph aspiration during backward beating, during forward pulses some hemolymph can enter the diastolic heart through the first ostia too, thus providing a lateral circulation in the thorax (Fig. 12). This explains why the forward pulse periods generally last longer than the backward pulse periods, without the problem of an over-accumulation of hemolymph in the anterior body. Even when the heartbeat changes from forward to backward pulse-wise (Fig. 7), the hemolymph ‘circulates’ in the anterior body.

*The possible role of abdominal movements and a modified pericardial septum in regulation of heartbeat reversal*

In Lepidoptera and Calliphora, the direction of heartbeat is influenced by the tension of the abdomen (Wasserthal, 1996; Wasserthal, 1999). In the present investigation it is observed that, when the abdomen is fixed, the *Drosophila* heart beats irregularly or changes its pulse direction with each pulse. It is concluded that in *Drosophila* too the abdomen plays a leading role in lowering the hemocoelic pressure by active expansion during retrograde beating of the heart and augmenting the pressure by contraction during anterograde beating, as in Lepidoptera and in Calliphora (Wasserthal, 1981; Wasserthal, 1982b; Wasserthal, 1996; Wasserthal, 1999; Wasserthal, 2003a). During anterograde pulses a positive hemocoelic pressure in the abdomen probably prevents the hemolymph from leaving the caudal opening. In addition, two large spongy cells on the rear of the second pair of ostia project deeply inward into the heart lumen (Miller, 1950). They probably prevent a back-flow towards the caudal opening during anterograde systole. By contrast, the heart can transport efficiently backward into the abdomen only if the hemolymph pressure in the abdomen is lower than the diastolic pressure inside the heart, otherwise hemolymph would be sucked back into the heart immediately after having left it. This mechanism may also influence the filling process of the anterior heart chamber directly or by support of the pericardial septum. It forms a muscular sheath with a cavity around the anterior heart chamber. If the internal hemolymph pressure by abdominal expansion during retrograde pulses decreases, the suction should dilate the pericardial septum and may thus explain the ‘diastolic volume increase’ observed by Dulcis and Levine (Dulcis and Levine, 2005), which does not occur in their bath preparation when retrograde pulses are stimulated by application of glutamate. The arrangement and attachment of the muscles of the pericardial septum at the metanotal cuticular ridge and at the ventral surface of the mesophragma suggest that their contraction increases the pericardial cavity and assists in distending the first heart segment. An active and important role of the pericardial septum can be deduced from the rich supply by the anterior transverse nerves. They form unique synaptic structures on its ventral longitudinal muscles (Dulcis and Levine, 2003). In addition, only the conical chamber receives input from the thorax *via* the median nerve. This suggests that the assumed pacemaker function of the anterior heart chamber

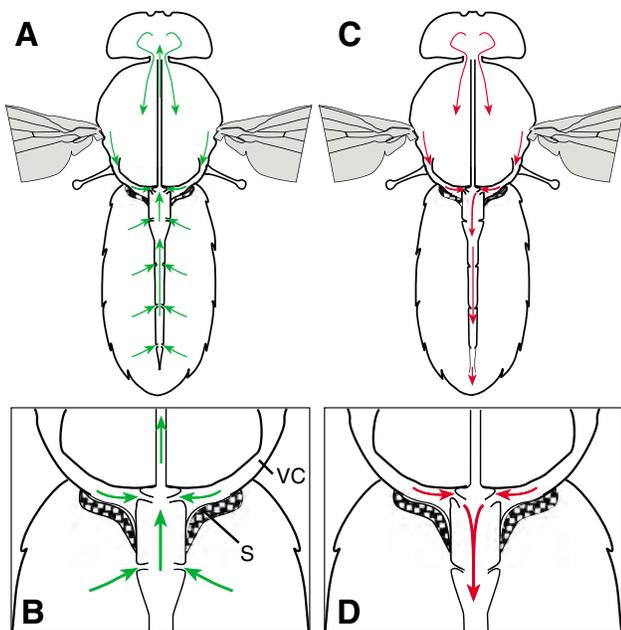


Fig. 12. Graph summarizing hemolymph circulation through the forward (A,B) and backward beating heart (C,D). (A,C) Overview; (B,D) detail, showing the central location of the anterior heart and its strategically favourable connection to the venous channels (VC). The lateral thoracic hemolymph flows through these channels directly to the first ostia. During diastole of the backward beating heart, thoracic hemolymph enters these anterior ostia exclusively. During forward beating, hemolymph is aspirated from the heart through all ostia, including the first ones. Thus a lateral circulation in the thorax is maintained independently of heartbeat direction. S, pericardial septum.

(Ritzki, 1978) is under neuronal control (Dulcis and Levine, 2003). Moreover, some coordination of abdominal movements with heartbeat reversals may be mediated by these nerves.

*Periodic hemolymph shift by heartbeat reversal and the physiological significance of having the hemolymph supply separated between thorax and abdomen in higher flies*

Adult flies are lightweight constructions adapted for flight with a reduced hemolymph content (Wigglesworth, 1963; Nicholson, 1976). The body in many flies is divided into an anterior and a posterior hemocoelic compartment separated by a huge pair of air sacs in the anterior abdomen (Faucheux, 1974). In *Calliphora* they function like a septum and facilitate an alternating periodic increase and decrease in hemolymph volume in both compartments by hemolymph shift with a compensatory ventilation effect upon the tracheal system (Wasserthal, 1999; Wasserthal, 2003a). In *Drosophila* without these large air sacs (Wigglesworth, 1950; Miller, 1950; Faucheux, 1974), the described venous channels and septum enable a similar periodic hemolymph shift. The hydraulic effects of the small hemolymph volume can be more effectively used by separation of both compartments. Periodic reduction of the hemolymph volume in the anterior body is compensated by an increase of the tracheal air sacs with consequential inspiration. The periodic tracheal volume changes in the head of *Calliphora* and *Drosophila* could be visualized by x-ray videographs at the ESRF-synchrotron (Wasserthal et al., 2006).

*Gene activation pattern and number of ostia*

The gene activation pattern in *Drosophila* heart morphogenesis has been elucidated including the ostial valve determination (Cripps and Olson, 2002; Gajewski et al., 2000; Popichenko and Paululat, 2004; Reim and Frasch, 2005). The embryological status shows seven pairs of ostial precursor cells. The anterior four pairs of ostia predetermined in the embryo are not manifested in the later larva, which possesses only three pairs. Until now, in adult *Drosophila*, only four pairs of heart ostia in total have been described, with the first pair in the second abdominal segment (Curtis et al., 1999). Preparations with seven double pairs of svp-expressing cells with two accessory terminal ones have been shown in wild-type *Drosophila* (Gajewski et al., 2000; Zaffran et al., 2002). With this newly described pair of imaginal heart ostia in the conical heart chamber there are in fact five pairs developed in the adult *Drosophila* heart tube. This is homologous to the situation in *Protophormia terraenovae* (Angioy et al., 1999) and in *Calliphora vicina* (Wasserthal, 1999). In *Calliphora*, with its very similar heart and a similar pattern of heartbeat reversals, additional terminal openings have been found in REM preparations (Wasserthal, 1982b; Wasserthal, 1999), and these have been confirmed for *Protophormia* in semithin sections (Angioy et al., 1999). The above-mentioned two svp cells at the end of the embryonic heart may be precursor cells for the excurrent openings at the caudal heart of adult flies, which are paired in *Calliphora* and *Protophormia* and unpaired in *Drosophila*. Documentation of a new anterior pair of ostia in the first abdominal segment of the conical chamber and of a caudal heart opening will be essential for reconstructing the

ontogenetic fate of predetermined heart tissue during metamorphic changes (Molina and Cripps, 2001).

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