The Journal of Experimental Biology 210, 2627-2636
Published by The Company of Biologists 2007
doi:10.1242/jeb.001644

Hemodynamics in the leech: blood flow in two hearts switching between two constriction patterns

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Accepted 15 May 2007

Summary

Two tubular, segmented hearts propel blood through the closed circulatory system of the medicinal leech and switch every 20–40 beats between two constriction patterns. We showed recently that within one heartbeat cycle, heart segments on one side constrict peristaltically rear-to-front ('peristaltic heart'), followed by nearly synchronous front-to-rear constrictions in the contralateral heart segments ('synchronous heart'). Using optical recordings from intact leeches, we now characterize the hemodynamic properties of the cardiac cycle of individual heart segments in different regions to ask whether the reversal of constrictions affects flow into, out of, and along the hearts. We measured total vessel capacity in corrosion casts and blood volume in individual heart segments of dissected leeches. We show that the peristaltic heart provides the propulsive force for forward and rearward flow and supplies the peripheral circulation through segmental efferent vessels. In comparison, the synchronous heart pumps less blood, most of which enters the segmental circulation. The heart sphincter, located in the posterior section of each heart segment, directs blood flow differently in the two modes. In the peristaltic heart, the sphincter prevents backflow and promotes longitudinal, forward flow while in the synchronous heart the sphincter restricts longitudinal, rearward flow and instead promotes flow into the segmental circulation. Blood is shunted *via* the contractile latero-dorsal arches from the dorsal intestinal vessel into the peristaltic heart in posterior segments 14 to 18. Switching between the two constriction patterns provides nutrient-rich blood to the vascular beds on both sides.

Supplementary material available online at
http://jeb.biologists.org/cgi/content/full/210/15/2627/DC1

Key words: invertebrate, circulation, cardiac cycle, leech, hemodynamics, blood flow.

Introduction

Circulatory systems need to meet the changing perfusion demands of different vascular beds. In vertebrates, with unidirectional flow in a closed circulatory system and a well-developed arterial tree, redistribution of blood flow is made possible by sphincters located at the entrance to peripheral vascular beds distal from the hearts. By contrast, blood distribution in open circulatory systems is accomplished by controlling cardio-arterial valves or sphincters located close to the heart at the entrance of entire arterial trees, as for example in many molluscs and arthropods (reviewed in McMahon et al., 1997). Segmented animals with a closed circulatory system such as annelids face yet a different challenge. Oxygenated and nutrient-rich blood needs to be distributed to multiple, parallel segmental capillary beds (integument, musculature, inner organs) and to serial vascular beds (nerve cord, intestine) with little wiggle room for redistribution to specific vascular beds. In the giant earthworm *Megascolides*, which has up to 1000 segments, circulation time increases from anterior to posterior, effectively dividing the animal into different circulation zones along the body axis, with the shortest turnover times in the region of the brain and reproductive organs (Jones et al., 1994).

In jawed leeches such as *Hirudo*, the focus of our study, the two lateral longitudinal vessels serve as hearts. They reflect the segmental body plan and are made of similar 'modules', with each heart segment having up to two contractile afferent vessels and one efferent vessel (Fig. 1) (Boroffka and Hamp, 1969). At any given time, the hearts on the two sides are coordinated differently along the body axis, with regular and precipitous switches between two modes of coordination (Thompson and Stent, 1976a). Our recent quantitative analysis of the constriction pattern on a segment-by-segment basis showed that the heart segments on the peristaltic side (termed the 'peristaltic heart') constrict rear-to-front while the heart segments of the contralateral, synchronous side (termed the 'synchronous heart') constrict front-to-rear with shorter intersegmental delays (Wenning et al., 2004a). Switches occur every 20–40 beats (Krahl and Zerbst-Boroffka, 1983; Thompson and Stent, 1976a; Wenning et al., 2004b). Intravascular systolic/diastolic pressures are about 6.7/0.5 kPa for the peristaltic heart and 3.3/0.5 kPa for the synchronous heart (Hildebrandt, 1988; Krahl and Zerbst-Boroffka, 1983). Although myogenic in nature, leech hearts are *de facto* neurogenic and require phasic input from 16 pairs of segmental heart motor neurons for

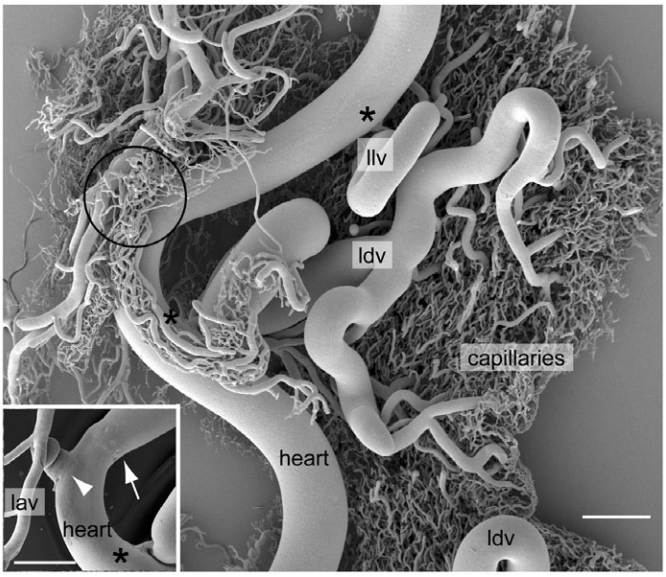

Fig. 1. Scanning electron micrograph in a corrosion cast to show the heart, its two afferent vessels, the latero-lateral (llv) and the larger latero-dorsal (ldv) vessels, and the efferent latero-abdominal vessel (lav, inset). The afferent vessels receive blood from the capillaries of the integument and body wall musculature and are contractile up to their first bifurcation. Inset: removing the capillaries (circle) reveals the latero-abdominal sphincter (arrowhead) and the bifurcation of the efferent latero-abdominal vessel. Just anterior is the heart sphincter of the next anterior heart segment (arrow). Due to the pressure necessary for the resin injection (see text), the valves between the side vessels and the heart (asterisks) are closed, as indicated by the constriction between the two vessels. Some capillary beds were trimmed for viewing purposes. Right body side, anterior to the top. Scale bars, 500 μm.

coordination, timing and switching between the peristaltic and the synchronous mode (Maranto and Calabrese, 1984a; Maranto and Calabrese, 1984b). The heart motor neurons are in turn driven by a well-studied heartbeat central pattern generator (for reviews, see Calabrese et al., 1995; Kristan et al., 2005).

The apparent flow reversal when switching from the peristaltic into the synchronous mode (Wenning et al., 2004a) prompted us to re-visit the hemodynamic properties of the leech's hearts with respect to the flow into and out of the segmental 'modules' and to longitudinal flow along the body axis. We measured vessel capacity using corrosion casts of the circulatory system. Measurements of vessel diameters *in situ* yielded information about blood volume in individual heart segments along the body axis. We used optical recordings in intact animals to avoid dissection, which causes ballooning and cessation of contractions of exposed blood vessels. We used juvenile leeches because their weak pigmentation provided good contrast for the flow of red blood. Except for shorter heartbeat periods than those of adult leeches (juveniles, 4.7±0.7 s; adults, 10.0±3.5 s), the constriction pattern as well as the switch dynamics are very similar (Wenning et al., 2004b). Optical recordings were used to characterize the cardiac cycles of individual heart segments and to assess volume differences between the peristaltic and the synchronous heart. Part of the results has previously been published in abstract form (Wenning and Calabrese, 2003).

Materials and methods

Adult leeches [either *Hirudo verbana* or *Hirudo medicinalis* (Siddall et al., 2007)] were obtained from commercial suppliers (Leeches USA, Westbury, NY, USA; 0.8–1.5 g) or, for the experiments carried out in Zürich (Switzerland), from a local pharmacy (2.0–3.0 g). Unfed juvenile leeches (2–6 months old, 100 mg) were kindly provided by W. B. Kristan and K. A. French (UCSD, San Diego, CA, USA). Leeches were kept in artificial pond water at 16°C. Leeches were cold anaesthetized and pinned through both the anterior and posterior sucker in a stretched position. Experiments were carried out at room temperature.

Corrosion casts of the vasculature

We assessed vessel capacity in corrosion casts of adult leeches using a polyurethane resin (PU4ii; vasQtec, Zürich, Switzerland) (Beckmann et al., 2003; Krucker et al., 2006). The resin was diluted with ethylmethylketone (30% w/v) to lower viscosity. Timely polymerization and minimal shrinking yield elastic casts that retain their original structure to facilitate post-casting tissue dissection and pruning.

A longitudinal slit through the body wall between segments 10 and 12 exposed the dorsal vessel. The body wall was forced apart and held in place with small hooks. The dorsal vessel was opened and a flexible polyethylene catheter (o.d. 80–150 μm) forwarded into the vessel lumen. To avoid rupture of the vasculature during pressure injection with the resin, we slowly injected 0.5–1 ml paraformaldehyde [4%, diluted 1:1 with leech saline (mmol l^{-1}): 115 NaCl, 4 KCl, 1.8 $CaCl_2$, 10 glucose, 10 Hepes buffer; pH 7.4] until the fixative returned to the injection site (1–2 min). Resin was injected for about 5 min using a perfusion pump set at 100–200 μl min^{-1}. In complete fills, the resin returned to the injection site and the tissue became rigid.

After polymerization (4–8 h), preparations were digested in 7.5% KOH (w/v; overnight at 55°C). Casts were thoroughly rinsed with water and freeze-dried. For inspection of inner surfaces, a segment of the cast was opened longitudinally and unfolded. Portions of the casts were processed for scanning electron microscopy (SEM) (Hitachi S4000, Naka, Japan) by sputtering with gold.

Measurements of segment length and vessel diameter in adult leeches

Segment length was measured in eight intact, moderately stretched leeches. In 19 freshly dissected leeches, we measured the diameters of the hearts and in some animals also the side vessels in maximal diastole and systole. To minimize dissection time and blood loss, we measured the heart segments in the anterior and posterior sections separately. Segment 10 was our reference with its end-diastolic diameter set at 100%.

Video imaging of intact juvenile leeches

In juvenile leeches, we video-imaged the constrictions of, and blood flow through, the hearts and their side vessels. The method and the analysis of the optical signals were described previously (Wenning et al., 2004a). In brief, leeches were

pinned through the anterior and the posterior sucker, ventral side up, in a stretched position. Imaging an entire juvenile leech took 10–45 min, capturing 3–6 segments at a time. Video clips were digitized (Imaging Workbench software, vs. 4; Axon Instruments Inc., Union City, CA, USA) for the automated analysis of vessel constrictions. Rhythmic filling and emptying of the vessels with red blood caused light intensity changes ('optical signals') in user-defined analysis windows drawn around desired sections of the blood vessels. Absolute values of the digitized signals depended on the analysis windows' size and on vessel visibility and were therefore not comparable between different animals. Data analysis was performed off-line using custom-made MATLAB software (Mathworks, Natick, MA, USA). We expressed time differences as a percentage of the heartbeat period (100% phase=heartbeat period).

To describe the cardiac cycle of an individual heart segment, we first determined the minimum (trough) and the maximum (peak) of the optical signal. The following points were then identified: start of diastole (=10% filled), maximal diastole (trough of the optical signal), the attainment of systole (here referred to as 'systole'), which was estimated best by the moment in time halfway between maximum diastole and the moment in time of the maximum growth of the optical signal corresponding to emptying, and the end of systole (=90% empty). '10% filled' and '90% empty' correspond to the same value of light intensity of the optical signal but not to the same point in time.

Data are from eight juvenile leeches. Four of those were quiescent, and recordings were stable long enough to cover at least one switch, enabling us to compare the end-diastolic volume and the volume pumped per cycle between coordination modes. We measured the end-diastolic volume as the maximal amplitude of the optical signal and the total volume pumped as the area under the curve between two systolic maxima using Clampfit (Axon Instruments Inc., Molecular Devices Corporation, Sunnyvale, CA, USA).

Statistics and nomenclature

Values are expressed as means of the averages of individual experiments ± s.d. Leeches have 32 segments, some of which are fused to form the head and tail brains. Segment #1 is assigned to the metameric body segment innervated by the most anterior (non-cephalic) ganglion of the ventral nerve cord, and segment #21 to the last metameric segment anterior to the tail brain.

Results

Vessel capacity and blood distribution

To assess the contribution of the different vascular beds to total capacity, we injected a fast-curing resin into the dorsal vessel, a superficial vessel on the low pressure side of the circulatory system (Fig. 2A; Fig. S1 in supplementary material). Of 25 casts, six gave complete fills. Sections of the casts were processed for scanning electron microscopy, revealing the major vessels and the dense capillaries of the inner organs and the body wall (Fig. 1, Fig. 2A; Fig. S1 in supplementary material). Two complete casts were used to dissect, and weigh, different vascular beds (outlined in Fig. 2A): (1) the contractile portion of the afferent vessels, (2) the two longitudinal lateral vessels, the hearts (segments 1–21), (3) the ventral and the dorsal vessel, (4) the small-caliber capillaries underneath the integument, the capillaries of both suckers and those of the body wall musculature, and (5) the capillaries of the inner organs (excretory organs, sex organs, intestine, dorso-ventral muscles). The capillary beds together made up about 57% of the total capacity. The contractile portions of the circulatory system, the afferent vessels and the two hearts, each held about 18%. Unlike in the living animal, both hearts were maximally filled. Paraformaldehyde injected into the dorsal vessel prevented rupture of the vessel walls during pressure injection of the resin. However, at the same time, the fixative likely stopped the regular heartbeat since the segmental ganglia – and hence the heartbeat central pattern generator – lie in the ventral vessel. Injection of the resin took several minutes (i.e. longer than a switch cycle) and filled all vessels maximally, with a bias towards the contractile vessels. For example, the ratio between the dorsal vessel and the hearts was smaller in the casts than in freshly dissected leeches as described below (casts, 0.5; dissected animals, 0.83; Fig. 2C).

Length and diameter of individual heart segments decreased in anterior and posterior segments. We used the segment length in moderately stretched, intact leeches (N=8) along the body axis between segments 3 and 18 to assess the length of the heart in that segment. Next, we measured the diameters of the hearts in maximum diastole (end-diastolic diameter) in freshly dissected adult leeches (N=19 animals) (Fig. 2C). Assuming a straight tube, we calculated the blood volume present at the end of diastole for individual segments using length and diameter. Using heart segment 10 as the reference, with its end-diastolic volume set to 100%, vessel capacity dropped to about 30% in the front and the rear (heart segments 3, 17 and 18; Fig. 2D). These measurements underestimate the true end-diastolic volume by about 20% because the hearts form a torturous line (Fig. 1). This arrangement is found in leeches of resting length and during swimming alike, providing the necessary slack for the change in the length with each contraction because the hearts' spindle-like muscle cells shorten as well as constrict (Maranto and Calabrese, 1984a). The end-diastolic diameters of the incoming and the single outgoing vessels in midbody segments 7–14 were (as a percentage of the corresponding diastolic heart) 60% (±17%; six animals) for the afferent latero-lateral vessel, 74% (±23%; three animals) for the afferent latero-dorsal vessel and 37% (±8%; five animals) for the efferent latero-abdominal vessel. The diameter of the dorsal vessel was 83% (±12%; four animals) of that of the heart (segment 10).

To estimate total blood volume in leeches, we calculated the volume in heart segment 10 (1.2±0.12 μl) using the end-diastolic heart diameter and the segment length of leeches of the same weight class (Fig. 2D). Second, using the average vessel diameters determined for each heart segment from the same animals, we calculated the blood volume of one lateral heart tube as 11.0 μl. Third, using the blood distribution in the different vascular beds determined from the corrosion casts (Fig. 2B), we calculated the total blood volume as being about 120 μl in leeches weighing 1.4±0.3 g, which translates into about 8–9% of the body mass. This value is an estimate; on the one hand, the contractile vessels are overfilled in the casts, while

on the other hand, the hearts' S-shape had not been taken into account when calculating the end-diastolic volume (see above).

Filling and emptying of a midbody heart segment

We constructed the cardiac cycles for individual heart segments. Fig. 3 shows a typical optical signal for midbody segment 10 and its cardiac cycle. The optical signal yielded the information for the period, filling, maximal diastole and emptying. The length of one cardiac cycle is set to 100%. Emptying took up about 20%, while filling took up about 30%.

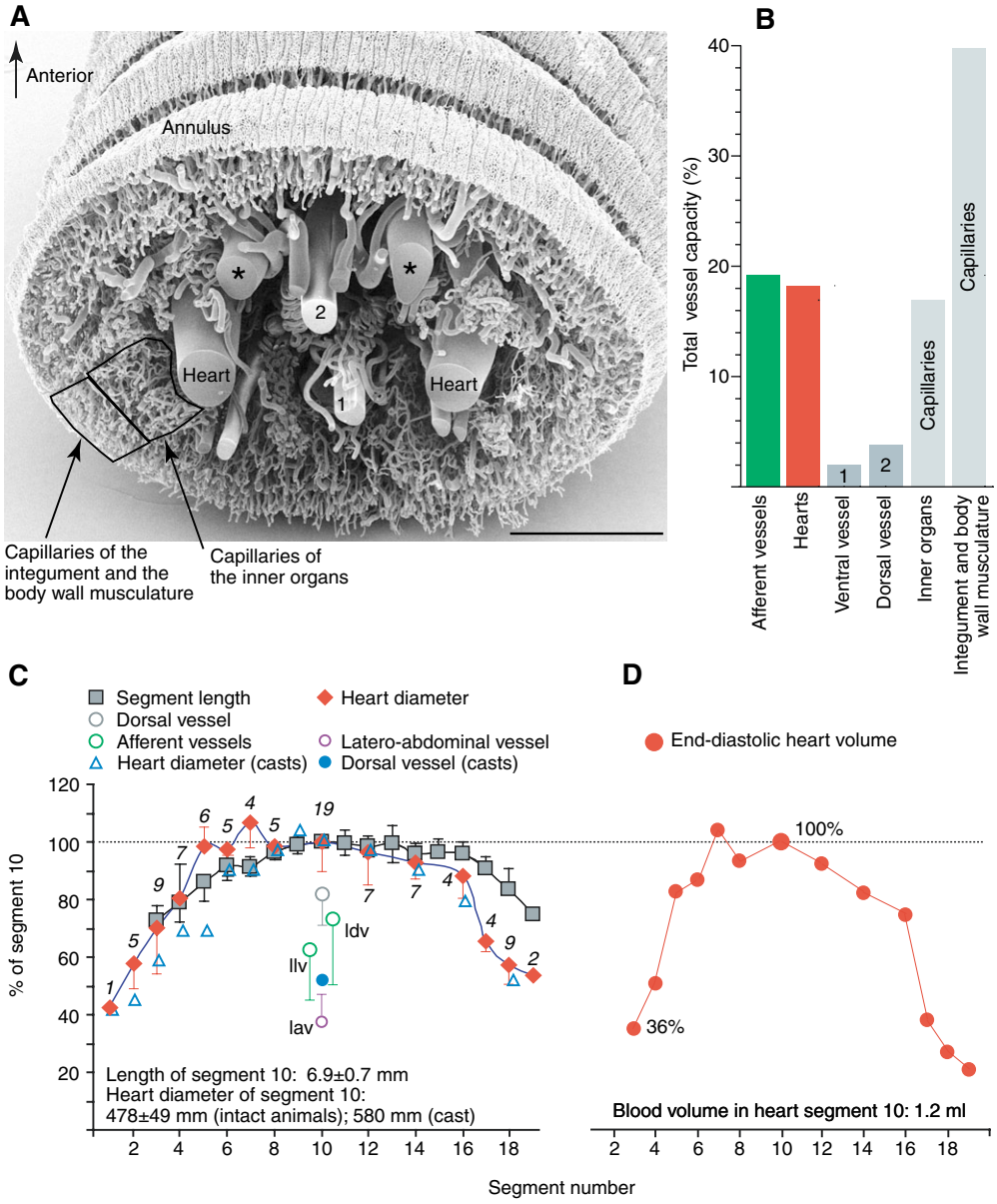

Fig. 2. Assessment of total vessel capacity in corrosion casts (A,B) and dissected animals (C,D). (A) Scanning electron micrograph of a transverse section through a corrosion cast near the segmental border of segments 4 and 5 to show the different vascular beds used to assess total vessel capacity: hearts; afferent latero-dorsal vessels (asterisks); ventral vessel (1; with the imprint of the connectives between the segmental ganglia); dorsal vessel (2); capillary beds of the integument and the muscular envelope and capillary beds of the inner organs (polygons). Note the small-caliber capillaries of the integument and the somewhat larger capillaries of the adjacent layer in the body wall musculature. Scale bar, 1 mm. (B) Blood vessel capacity was assessed by dissecting and weighing the vascular beds of completely filled corrosion casts. The graph shows the average of two casts. About 60% of the total blood volume is stored in the capillary beds of the inner organs and the body wall and its musculature. (C) Segment length and the end-diastolic diameter of individual heart segments vary along the body axis. Segment length was measured in eight intact leeches (grey squares). The diameter of individual heart segments (blue triangles) and of the dorsal vessel in segment 10 (blue filled circle) were measured in one cast. The end-diastolic diameters of the hearts (red diamonds; number of measurements in italics), the afferent vessels (green circles; ldv, latero-dorsal vessel; llv, latero-lateral vessel), the latero-abdominal vessel (purple circle, lav) as well as the diameter of the dorsal vessel (grey circle) were measured in freshly dissected leeches. Values are plotted as a percentage of segment 10 (means ± s.d.). Absolute values for segment 10 are given on the graph. (D) Using segment length and the end-diastolic diameter, the end-diastolic volume was calculated for each heart segment assuming a straight cylinder. All data from adult leeches.

After emptying, the heart appeared about 10% filled for half of the cardiac cycle before filling started again.

Heart segments 4–18 have two afferent vessels (the latero-lateral and latero-dorsal vessel, respectively), segments 2, 3 and 19 have one (Boroffka and Hamp, 1969). The afferent vessels deliver oxygenated blood from the integumental capillaries into the heart, are contractile and are on the low pressure side of the circulatory system (0–0.8 kPa) (Hildebrandt, 1988) (Fig. 1). Their orifices have (passive) valves that are pushed back when the pressure in the heart overcomes that of the afferent vessels (Hammersen et al., 1976) (see closed valve in Fig. 1). The non-muscular efferent latero-abdominal vessel serves the nephridia and the ventral musculature (Fig. 1; Fig. S1 in supplementary material). The neural network of the heart extends to the initial, muscular part of the latero-abdominal vessel (Wenning and Cahill, 1986), which serves as a sphincter (Fig. 1, inset).

The afferent vessels constrict before the hearts (Boroffka and Hamp, 1969; Hammersen et al., 1976; Hildebrandt, 1988). We quantified the difference between systole in the hearts and in the afferent vessels (see Materials and methods for the definition of systole and Fig. 3). Systole in the hearts was assigned 0% phase. We determined systole in the heart and the afferent vessels between segments 8 and 16 from six intact, restrained juvenile leeches (5–15 heartbeat cycles per segment, 1–4 segments per animal). On average, systole of the afferent vessels led heart systole by –17.3±3.6% (Fig. 3; horizontal dotted bars) with no difference in the two coordination modes.

Fig. 4A,B shows the latero-dorsal vessel and the heart in segment 15 and their constriction patterns. In these posterior

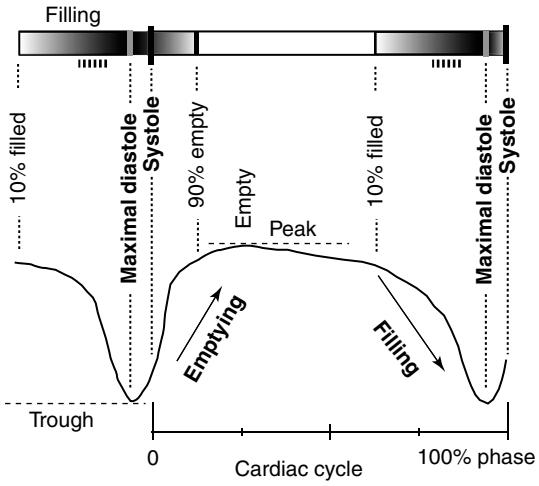

Fig. 3. Hemodynamic properties of the cardiac cycle (bar) in one heart segment to show the timing of filling and emptying (example: heart segment 10). The optical signal (black line) reflects the rhythmic filling (downward deflection) and emptying (upward deflection) of the heart segment and yields the information for the start of diastole (=10% filled), maximal diastole (trough), the attainment of systole ('systole'; see Materials and methods for definition) and the end of systole (=90% empty). Phase relations were based on systole set at 0% phase. The period of one cardiac cycle was normalized to 100% phase (4.7±0.7 s in juvenile leeches). Systole of the afferent vessels and the closure of the efferent latero-abdominal sphincter occurred at the same time, just before maximal heart diastole (horizontal dotted bars).

segments, latero-dorsal vessels fuse and form the latero-dorsal arches (Boroffka and Hamp, 1969). Images taken at four points (a–d) of the cardiac cycle (Fig. 4C,D) illustrate the sequence of events. The first image (a) shows a filled latero-dorsal vessel and a partially filled heart. The latero-dorsal vessel entered systole first, rapidly filling the heart (a–b). When the heart entered systole, the valve closed, preventing backflow. During heart systole (c), the latero-dorsal vessel was already filling again (c–d).

Blood leaves the heart and enters the segmental circulation through the latero-abdominal vessels present in segments 3–18. Fig. 5 shows optical recordings from the hearts, the efferent latero-abdominal sphincters and the vessels in segment 11 on both sides. In both coordination modes, the latero-abdominal sphincter closed briefly and transiently before heart systole. Closure of the sphincter occurs at about the same time as systole in the afferent vessels (data not shown).

We conclude that both the peristaltic and the synchronous heart receive blood from the afferent vessels and deliver blood through the efferent vessel into the segmental circulation.

Flow along the body axis in the peristaltic and the synchronous heart

The relative progression of systole between heart segments along the body axis did not vary with period and enabled us to average between animals (Wenning et al., 2004a). The phase diagram in Fig. 6 shows the intersegmental and side-to-side coordination of heart segments 3–18 for the peristaltic (magenta) and the contralateral synchronous (blue) heart. Within one heartbeat cycle, heart segments on both sides complete their cardiac cycles.

Starting with a nearly simultaneous systole in heart segments 17–15 on the peristaltic side (set arbitrarily to 0% phase in Fig. 6), systole traveled rear-to-front with an average segmental delay of 4.6% within about 60% of one heartbeat cycle. Heart segments anterior to the one in systole are in diastole, allowing blood to flow forward along the heart tube. Blood entering from the afferent vessels adds volume while the forward progressing constrictions add momentum. The afferent vessels constrict before the next posterior segment (afferent vessels, –17% phase; next posterior segment, –4.6% phase on average). Thus, filling from the afferent vessels precedes filling from systole in the adjacent posterior heart segment. Forward flow is aided, and rearward flow prevented, by the heart sphincter in the posterior section of each heart segment (Fig. 1, inset), which occludes the lumen at the onset of constriction. The sphincter is a thickening in the muscular vessel wall due to asymmetries in the orientation of the spindle-shaped heart muscle cells (Maranto and Calabrese, 1984a). As a consequence of this flow pattern, heart segments 3 and 4 were filled for most of their cardiac cycles while midbody segments remained partially filled right after their systole for about 50% of the cardiac cycle (Fig. 6; compare segments 4 and 10). Posterior heart segments fill somewhat earlier in their cardiac cycle (shown for segment 16 in Fig. 6). Thus, with each heartbeat, the peristaltic heart delivers the equivalent of several heart segments into the head region (anterior sucker and head brain).

Systole in the synchronous heart (Fig. 6) traveled rearward, taking up about 30% of the heartbeat cycle period. Constrictions

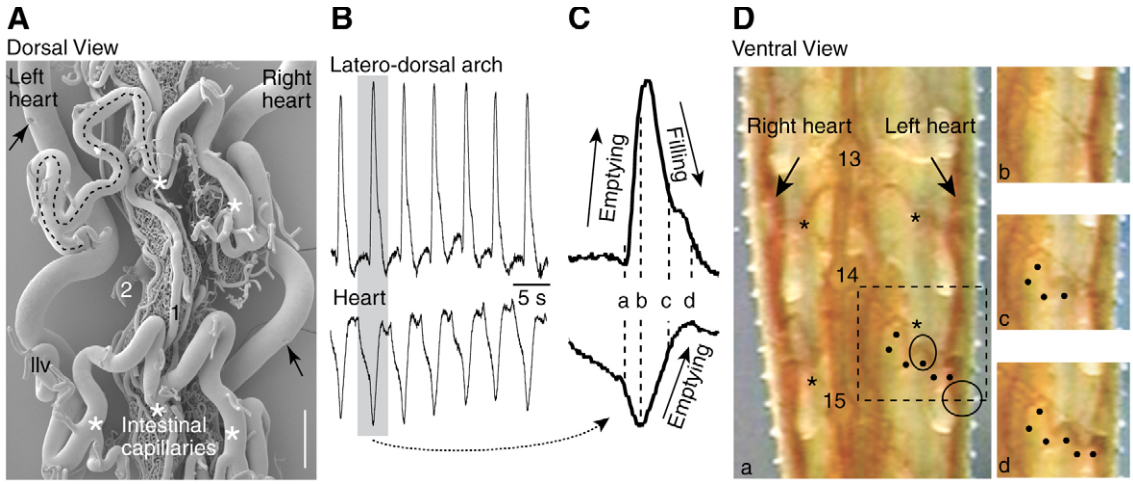

Fig. 4. Filling of the hearts by segmental afferent vessels. (A) Scanning electron micrograph of heart segments 15 and 16 in a corrosion cast in the intestinal region. Left and right latero-dorsal vessels fuse (fusion area; small asterisk) above the dorsal intestinal vessel (1) and form the latero-dorsal arches (asterisks). The dotted line follows the left latero-dorsal arch of segment 15 from its fusion point with the right arch to its valve at the heart. In comparison, the latero-lateral vessel (llv) is smaller and shorter. Its insertion point into the hearts is seen in two locations where the vessel broke away (arrows). Note the ventral vessel (2) with the enlargement for the ganglion of segment 15. Most capillary beds (integument, muscles, testes, nephridia) were removed. Scale bar, 1 mm. (B) Optical recordings from the latero-dorsal arch and the heart in the left hemisegment 15 (synchronous mode). In this preparation, the latero-dorsal arch constricted before the heart with an average phase difference of –23±3% (14 consecutive beats). (C) A single cardiac cycle (shaded area in B) is enlarged and shows the time points of the consecutive images of D. (D) Ventral aspect of segments 12–15 in the same animal as in B showing both hearts (arrows) and the latero-dorsal arches (asterisks). Analysis windows were drawn around the left heart of segment 15 and the latero-dorsal arch for optical recordings (rectangle in a). Consecutive images (a–d) show the timing of the constrictions of the latero-dorsal arch (dots) and the heart for the cardiac cycle enlarged in C. (a) Latero-dorsal arch filled, heart partially filled; (b) constriction of the latero-dorsal arch (no dots) fills the heart to maximal diastole; (c) heart in systole; latero-dorsal arch fills; (d) heart empty; latero-dorsal arch filled. Optical recordings are from intact juvenile leeches. Anterior is to the top.

started in the anterior heart segments with an average intersegmental delay of –2.7% between heart segments 3 and 13 (range, 0.3 to –3.8%) (Wenning et al., 2004a). Due to these short intersegmental delays of systole between adjacent segments, rearward flow is expected to be restricted. Importantly, the position and the role of the heart sphincter reverse (Fig. 1). The sphincter is now in front of the traveling blood column and effectively blocks rearward flow when that heart segment constricts. Indeed, the prepulse in systolic pressure, indicative of filling from the adjacent heart segment, disappears upon switching into the synchronous coordination mode (Krahl and Zerbst-Boroffka, 1983; Wenning et al., 2004a). Since the synchronous heart is not filled from adjacent, i.e. anterior, segments, it should carry less blood. We tested this hypothesis by measuring the end-diastolic volume (i.e. the peak amplitude) and the total pumped volume (i.e. the area from peak to peak) of the optical signal. Two conditions had to be met for a meaningful analysis. First, animals had to be quiescent since movements distort the optical signal and, second, recordings needed to span at least one switch in coordination mode to determine the relative volume change, preferably in both hearts of a given segment since the absolute values of the optical signal depend on the analysis window size and the visibility of the heart in that segment of that animal. Of the eight juveniles used in this study, four met these requirements.

Optical recordings from two different animals are shown in Fig. 7 for anterior (A) and posterior (B) heart segments. Volume differences were largest in midbody segments 5–10, where the volume pumped during one cardiac cycle in an individual heart segment in the synchronous coordination mode was between 50 and 70% of that in the peristaltic mode, and the end-diastolic volume was between 80 and 50% of that in the peristaltic mode (4–15 beats per coordination mode in 2–8 segments per animal; Fig. 7C) with somewhat smaller differences in anterior and posterior segments. Heart segment 3 on the synchronous side is in diastole when the peristaltic heart constricts and may receive blood directly (i.e. not only *via* the afferent vessels) from the head region, decreasing the volume difference between the two modes. However, the smaller diameter of heart segments 3 and 4 makes the optical signal more susceptible to noise. In segments 17 and posterior, not much blood from adjacent posterior segments contributes to filling because of their almost simultaneous constriction and their taper (Fig. 2C, Fig. 6), and blood volume should be similar on both sides. Indeed, there is no difference in the ratio of the pumped volume (101±5%), and the end-diastolic volume in the synchronous heart is now 89±11% of that of the peristaltic heart (e.g. segment 16 in Fig. 7B,C).

We conclude that the peristaltic heart propels blood from the rear to the front within one heartbeat cycle and that the synchronous heart carries less blood than the peristaltic heart and that longitudinal flow is restricted.

The role of the latero-dorsal arches

The latero-dorsal vessels in segments 14–18 extend and fuse, retaining their muscular envelopes over their length, forming the so-called latero-dorsal arches (Fig. 4A) (Boroffka and Hamp, 1969). In addition to receiving oxygenated blood from the

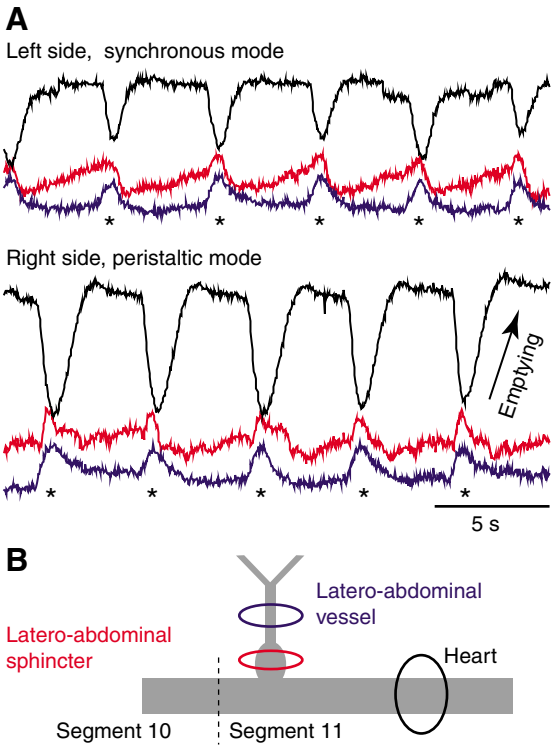

Fig. 5. (A) Optical recordings from both hearts (black) in segment 11 and from the latero-abdominal vessels (blue) and sphincters (red). The latero-abdominal sphincters close briefly just before maximal diastole (asterisks), causing a simultaneous interruption of flow in the vessels. (B) Position of the analysis windows around the hearts and the latero-abdominal sphincter and vessel.

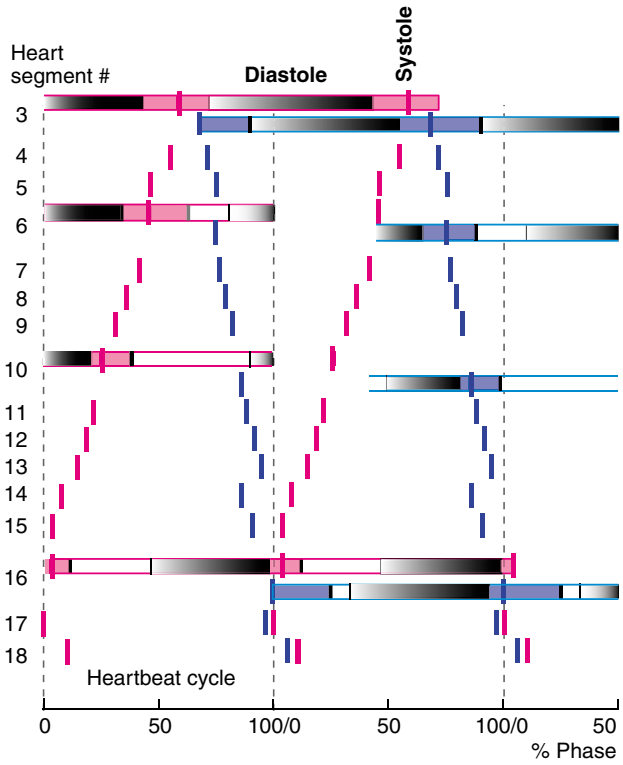

Fig. 6. Phase diagram to illustrate the intersegmental and side-to-side coordination of systole (vertical bars) in heart segments 3–18 for the peristaltic (magenta) and the synchronous (blue) modes. Data are duplicated and shifted by 100% to illustrate the side-to-side coordination over two heartbeat cycles [values for systole from Wenning et al. (Wenning et al., 2004a)]. Complete cardiac cycles (bars) are shown for segments 3, 6, 10 and 16 (labeling as in Fig. 3). Within the period of one heartbeat cycle, the heart segments on both sides completed their individual cardiac cycles. Starting with a nearly simultaneous systole in segments 17–15 (set to 0% phase) in the peristaltic heart, systole traveled rear-to-front within 60% of the heartbeat period. In the synchronous heart, systole traveled front-to-rear to segment 17 within about 30% of the heartbeat cycle. Note that of the 32 heart segments, only a fraction are in systole at any given time; the others, in diastole, may serve as a conduit (see text).

integument, they collect nutrient-rich blood from the intestine. As shown here, these arches provide a shunt between the dorsal vessel (which gives rise to the major intestinal vessels) and the hearts. Moreover, because the side-to-side phase differences between heart systole progressively decrease with a small but consistent phase advance of the synchronous heart (Fig. 6), there is a bias towards filling the peristaltic heart through the arches on both sides. Fig. 8A shows optical recordings from both hearts in segment 14 and from two points of each of the corresponding latero-dorsal arches across a switch in coordination mode (analysis windows outlined in Fig. 8B). Systole of the arch on the synchronous side coincides with filling of the peristaltic heart, followed by further filling from the ipsilateral, peristaltic side of the arch. As in the side vessels of more anterior segments, the constriction of the arches is strictly coordinated with the constriction of the ipsilateral heart segment.

Discussion

Within one heartbeat period, all segments of the two lateral heart tubes of the leech consecutively complete their individual cardiac cycles (Fig. 6). Rear-to-front progression of systole in the peristaltic heart takes 60% of the heartbeat period; front-to-rear progression of systole in the synchronous heart needs only 30%. While the two hearts beat out of phase in midbody segment 9, side-to-side differences decrease at the anterior and posterior ends. This constriction pattern has four implications. First, the two hearts operate sequentially, and heart segments not in systole support flow. Second, slow progression of systole in the peristaltic heart promotes longitudinal (forward) flow, while rapid progression of systole in the synchronous heart – especially between heart segments 5–13 – discourages longitudinal (rearward) flow and promotes segmental circulation. Third, blood volume should be lower in the synchronous mode (Fig. 7). Fourth, the deviation from an antiphasic heartbeat in the anterior and posterior region may encourage unidirectional flow through anastomoses and shunts instead of blood merely sloshing between right and left.

An intriguing feature of leech circulation is the asymmetry in the flow pattern, with regular, reciprocal and precipitous switches between rear-to-front (peristaltic) and a steeper front-to-rear (synchronous) wave of constrictions. Reversal in blood flow has been observed in other animals such as tunicates (Kriebel, 1968) and, prominently, in adult holometabolous insects. Here, alternation of flow direction in the dorsal heart is

thought to facilitate the perfusion of different vascular beds (wings, abdomen, thorax) and to be important for temperature control (Smits et al., 2000). In the moth *Manduca*, cardiac reversal relies on the alternation of pacemaker dominance of two separate pacemakers for forward and rearward flow (Dulcis et al., 2001). Leech heartbeat is shaped by a single central pattern generator. While temperature regulation is presumably unimportant in an aquatic species, the high degree of segmentation requires a balance between perfusion of segmental vascular beds and the distribution of nutrients along the body axis.

Based on pressure recordings in the hearts and the latero-abdominal vessels, Hildebrandt concluded that only the synchronous heart delivered blood into the segmental circulation, and less significant amounts were to leave the peristaltic heart (Hildebrandt, 1988). This division of labor suggested that the segmental capillary beds would receive only part-time nutrient-rich blood provided by the peristaltic heart and, importantly, would require separate neural control of the latero-abdominal sphincters in the two modes, for which there is no evidence (Maranto and Calabrese, 1984a; Maranto and Calabrese, 1984b). Regardless of the coordination mode, the sphincters close briefly just before the hearts enter systole (Fig. 5), suggesting that both hearts serve the peripheral circulation. However, since longitudinal flow in the synchronous heart is restricted due to the location of the heart sphincters and the fast progression of systole, blood volume is lower in the synchronous heart, most of which may be forced into the segmental circulation.

The afferent vessels constrict before the hearts, ensuring their timely filling. Like the hearts, they seem to be innervated by the segmental heart motor neurons since intracellular recordings showed that each motor neuron burst elicited plateau and excitatory junction potentials in the muscle cells of the afferent vessels (Wenning and Calabrese, 2003) (A.W., unpublished observations). Systole of the afferent vessels is tied to that of the hearts, as seen, for example, across a switch with its 'double beat' in the left hemi-segment 14 (Fig. 8). Yet, despite simultaneous excitation, systole in the afferent vessels precedes heart systole (Figs 4, 8), presumably because the ongoing diastolic filling of the hearts in conjunction with their larger diameter delay constriction (Fig. 2C). Since the optical recordings signal 'empty' or 'full' – and not contraction force – they will display this delay as a delay in emptying.

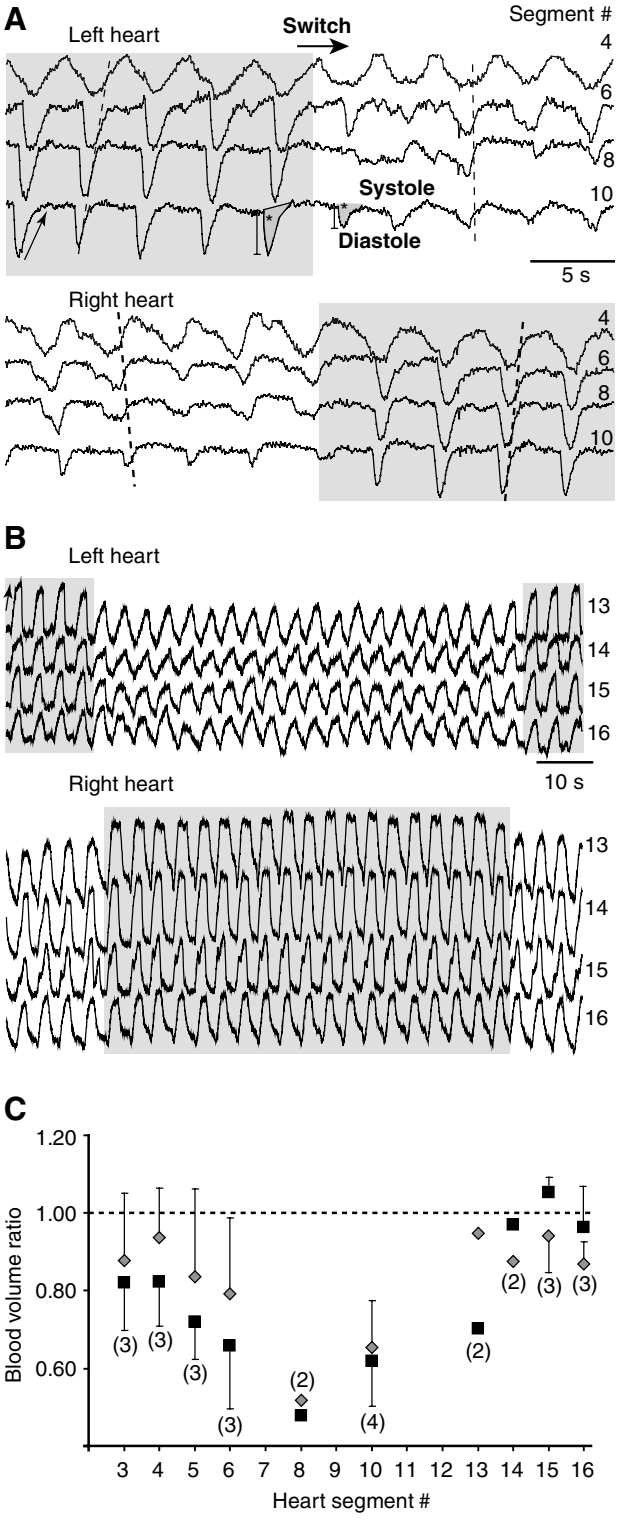

Fig. 7. Long-term optical recordings were used to assess volume differences in the hearts with respect to coordination mode. Recordings are from heart segments 4, 6, 8 and 10 (A) and from heart segments 13–16 (B) on both sides (peristaltic mode, shaded boxes; synchronous mode, no shading). Small arrows denote emptying (A, left heart, segment 10; B, left heart segment 13). In the top panel, dotted lines ease visualization of the characteristic intersegmental phase differences in the two modes. We assessed the end-diastolic volume as the maximum amplitude of the optical signal and the pumped volume during one cardiac cycle as the area under the curve (shaded areas in A, left heart). Heart segments 4–10 carry less blood in the synchronous mode than in the peristaltic mode. (B) Two switches in coordination mode are shown for posterior heart segments to emphasize the regular timing of constrictions as well as the precipitous and reciprocal switches in these quiescent leeches (different animal from that in A). Note that the posterior heart segments constrict nearly simultaneously (see text and Fig. 6). The differences in pumped and end-diastolic blood volume were less obvious in segments 14–16. (C) Ratio of the total blood volume pumped (squares) and the end-diastolic volume (diamonds) in the synchronous *vs* the peristaltic coordination mode from four animals. A ratio of 1 (horizontal dotted line) indicates no volume difference between coordination modes. Values are means ± s.d., with the number of preparations for each segment given in parentheses.

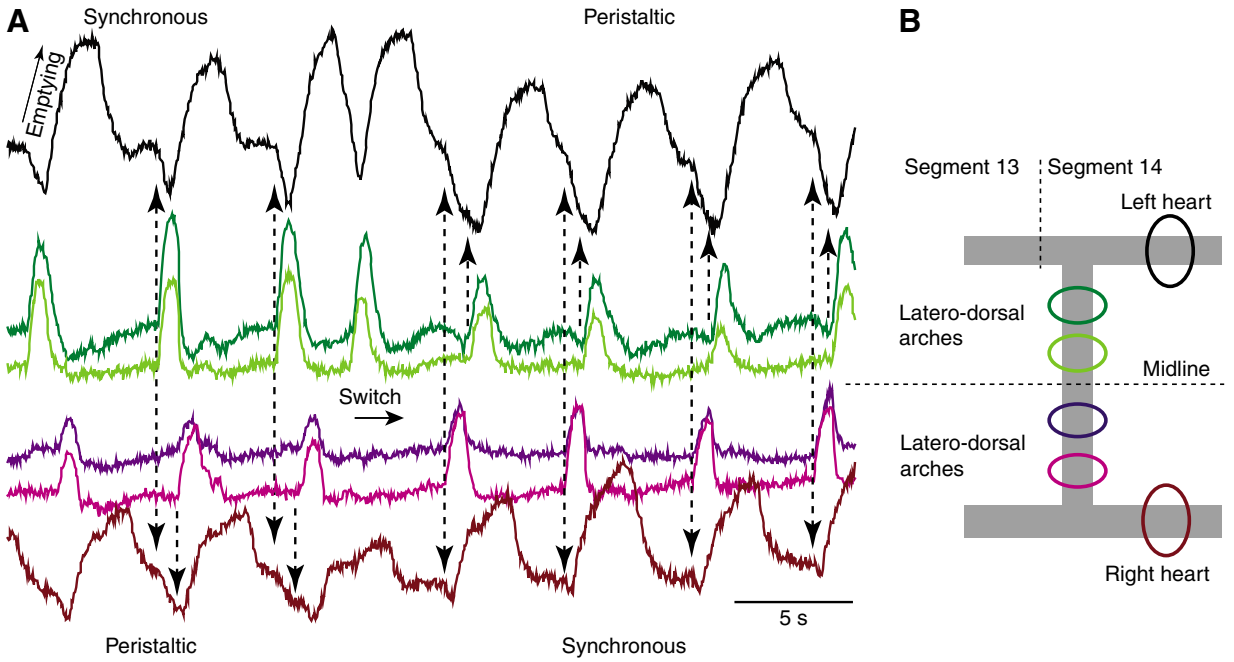

Fig. 8. (A) The latero-dorsal arches shunt blood from the dorsal vessel to the hearts, with a bias towards the peristaltic heart. Optical recordings are from segment 14 from the left (black line) and right (brown line) heart and from the latero-dorsal arches in the left and right hemisegment. (B) Analysis windows drawn around the vessels and the color code of the optical signals. Initially, the left heart is in the synchronous mode. Systole of the latero-dorsal arches in the left hemisegment (double-headed arrow), coincides with filling of both hearts (rapid downward deflection). After the switch, the now peristaltic left heart fills when the arches on the contralateral (synchronous) side enter systole (double-headed arrow). Conversely, systole of the latero-dorsal arches in the right hemisegment (double-headed arrows) after its switch into the synchronous mode coincides with filling of the contralateral, now peristaltic, heart. Additional filling occurs in the peristaltic heart when the latero-dorsal arch in its own hemisegment enters systole (arrows) but note that systole in the arches on the peristaltic side occurs during systole of the synchronous heart and thus is unable to contribute to its filling.

In segments 14–18, the afferent latero-dorsal vessels form arches that shunt blood from the dorsal vessel to the hearts (Fig. 8). Heart systole on the synchronous side is leading that on the peristaltic side by a decreasing but consistent margin (Fig. 6). These small side-to-side phase differences support unidirectional shunting towards the peristaltic heart because systole in the arches on the peristaltic side occurs during systole of the synchronous heart. Shunts allow blood to return to the heart faster, enhancing central circulation, and are common in highly segmented animals (Jones et al., 1994). In leeches, these posterior shunts provide an additional bonus because ingested blood is stored in a large crop spanning the entire animal, with the intestine confined to segments 14 and posterior. Shunting blood across the arches into the peristaltic heart facilitates the flow of nutrient-rich blood to anterior segments yet still allows perfusion of the posterior sucker and the tail brain.

Blood flows rearward in the dorsal and ventral vessel. A single dorsal vessel forms in segment 3 (Boroffka and Hamp, 1969) while the ventral vessel forms around the brain and the subesophageal ganglion and encloses the chain of segmental ganglia (Fig. 2A; Fig. S1 in supplementary material). In the front segments, there are anastomoses between the ventral and the dorsal vessel. In time-lapse videos, Hildebrandt showed that rearward flow in the dorsal vessel is pulsatile and flow oscillates between ~0.5 and 5 mm s^{-1} with the period of one cardiac cycle (Hildebrandt, 1988). Reasoning that the front heart segments may not discharge enough blood to fill the dorsal and the ventral vessel and that the highest pressure in the dorsal vessel, recorded in segment 10, coincides with the pressure peak in the synchronous heart, recorded in segment 6, Hildebrandt concluded that the dorsal vessel is filled mainly by the synchronous heart *via* the latero-abdominal vessels and the segmental capillary beds. We find it difficult to envision that pulsatile flow and pressure (between 0.9 and 1.9 kPa) (Hildebrandt, 1988) are sustained after blood has passed through several capillary beds. We show that only the peristaltic heart delivers blood to the head region (Fig. 6) and propose that the dorsal and the ventral vessel are filled predominantly by the peristaltic heart. Larger phase lags and the supportive action of the heart sphincter allow the peristaltic heart to propel and discharge an amount of blood into the head region equivalent to the volume of multiple heart segments in one peristaltic wave of systoles (minus the blood exiting into the segmental circulation). From the peristaltic heart, blood flows through numerous anastomoses into the dorsal and ventral vessel. This scenario explains the pulsatile flow and the oscillating pressure pulses observed in the dorsal vessel (Hildebrandt, 1988).

Regulation of leech heart performance

Any perturbations that affect the heart rate and/or switching must do so by altering the properties of the neurons constituting the heartbeat central pattern generator, which controls the 16 pairs of segmental heart motor neurons (Calabrese, 1977; Gramoll et al., 1994; Thompson and Stent, 1976b). Heart rate

is inversely related to temperature and can be modulated by stimulating identified neurons (Arbas, 1984; Arbas and Calabrese, 1990). Peptides (e.g. FMRFamide, myomodulin) increase the heart rate through interaction with the pattern generator (Kuhlman et al., 1985; Masino and Calabrese, 2002; Tobin and Calabrese, 2005). Leeches recover from prolonged periods of hypoxia (72 h) (Schmidt and Zerbst-Boroffka, 1993) and lower their heart rate in response to lower ambient oxygen (Davis, 1986).

Phase relations are invariant across changes in burst period in the entire system – from pattern generator to heart constrictions (Norris et al., 2006; Wenning et al., 2004a). Since the contractile vessels of one hemisegment share the excitatory drive, the sequence of events – afferent vessel constriction, sphincter closure, heart systole – is fixed and not coordination mode-specific. So far, all work on the circulatory system has been done in quiescent, restrained leeches. It will be interesting to study whether and, if so, how hemodynamics are modified to meet different metabolic demands such as in locomotion and feeding. Sustaining flow through the capillary beds (Fig. S1 in supplementary material) – some of them in series – as well as avoiding pooling, and stagnant anoxia are challenges that leeches might meet using behavioral responses by swimming a few lapses or doing a few stretches.

The leech hearts' constriction pattern is bilaterally asymmetric, but both hearts perform each task – albeit at different times. Both hearts serve the segmental circulation, but the peristaltic heart additionally provides the propulsive force for longitudinal flow, forward in the heart and rearward in the dorsal and ventral vessel. The heart segments' design and the different intersegmental coordination of constrictions rather than separate (neural) control of the sphincters allow the division of labor between the peristaltic and synchronous heart. Regular switching between coordination modes may prevent asymmetries in volume distribution and ensures flow of nutrient-rich blood from the intestinal region to anterior vascular beds on both sides.

We thank Dr Ronald L. Calabrese for his generous support and many discussions. Dr Andrey Olipher (Calabrese lab) modified the MATLAB code. Drs Irene Zerbst-Boroffka and Darrell R. Stokes provided valuable comments on the manuscript. Supported by Novartis (37202001 to E.P.M.) and NIH (NS24072 to R. L. Calabrese).

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