Heartbeat Control in Leeches. I. Constriction Pattern and Neural Modulation of Blood Pressure in Intact Animals

Angela Wenning, Gennaday S. Cymbalyuk, and Ronald L. Calabrese

Department of Biology, Emory University, Atlanta, Georgia 30322

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INTRODUCTION

Stereotyped rhythmic movements, particularly those for continuous autonomous functions like breathing and neurogenic heartbeat (Calabrese and Feldman 1997), are attractive objects for study of how the nervous system programs movement. These movements are driven by central pattern generators that operate without the strong cycle-to-cycle sensory feedback observed in pattern generators for locomotor movements (Pearson and Ramirez 1997). Thus understanding the mechanisms for rhythmicity, timing, and phasing in these central pattern generators may provide greater insight into the control of movement than for other pattern generators.

Heartbeat in medicinal leeches is an ongoing motor pattern driven by a well-studied central pattern generator (Calabrese et al. 1995; Hill et al. 2002, 2003). The two lateral vessels along the body axis serve as hearts and are made of similar segmental “modules” (Fig. 1) (Boroffka and Hamp 1969). Heartbeat in the leech is ongoing but not monotonous. Impedance measurements of the hearts in reduced preparations (Thompson and Stent 1976a) and intravascular pressure recordings in minimally dissected animals (Krahl and Zerbst-Boroffka 1983) revealed two different coordination modes. At any given time, one heart produces a frontward peristaltic wave with high systolic pressure (50 mmHg), while the contralateral heart produces nearly synchronous contractions with lower systolic pressures (20 mmHg; Fig. 1). Switches between these coordination modes are precipitous and reciprocal. Thus the same heart performs different tasks and switches between them.

In this and the accompanying paper, we describe experiments aimed to understand to what extent heart performance, specifically the intersegmental coordination of the heart constrictions and the pressure profiles typical for the two coordination modes, is governed by the heartbeat central pattern generator. Leech hearts are myogenic but require phasic input for coordination and timing from the heart motor neurons (Maranto and Calabrese 1984b; Thompson and Stent 1976a). Additional input comes from the modulatory heart accessory neurons, which increase the beat tension of hearts in reduced preparations (Calabrese and Maranto 1984; Maranto and Calabrese 1984a,b). The leech heartbeat central pattern generator, a well-characterized network of 14 identified interneurons, determines the activity pattern of the heart motor and modulatory neurons (Calabrese et al. 1995; Hill et al. 2002, 2003).

In this first paper, we describe the constriction pattern of the two hearts in intact, restrained leeches using imaging techniques. We also describe the time relation between the onset of heart systole and the corresponding heart motor neuron burst using simultaneous recordings from the heart motor neurons and imaging of the hearts. Finally, we revisit the role of the heart modulatory neurons in shaping the different blood pressure profiles characteristic for the two coordination modes by simultaneous intravascular pressure recordings in the lateral hearts and intracellular recordings from a heart modulatory neuron in minimally dissected leeches. These experiments provide the necessary information for assessing the role of the fictive motor pattern, described in the accompanying paper (Wenning et al. 2004), in shaping the constriction patterns of the hearts in the intact animal.

Part of the results appeared in abstract form (Wenning and Calabrese 2002; Wenning et al. 2000).
METHODS

Adult leeches (*Hirudo medicinalis* L.) were obtained from commercial suppliers (Leeches USA, Westbury, NY; Carolina Biological, Burlington, NC). Unfed juvenile leeches (2–4 mo old) came from a colony in San Diego, CA (courtesy of W. B. Kristan and K. A. French). Leeches were kept in artificial pond water at 16°C. For all preparations, leeches were cold anesthetized and pinned through both the anterior and posterior sucker in a stretched position. All dissections (described individually in the following text) were completed within 60 min. Experiments were carried out at room temperature (20–22°C).

Video imaging and analysis of heart constrictions

Either juvenile (40–100 mg) or small adult leeches (0.5–1 g) provided the intact preparations for video-imaging the heart constrictions and blood flow. Leeches were submerged in ice-cold artificial pond water. They were pinned through the anterior and posterior sucker, ventral side up, in a stretched position (20–35 mm length for juveniles, 80–100 mm for adults) in a silicone elastomer (Sylgard)-lined dish. To improve imaging, juvenile leeches were flattened by placing a cover slip over the whole animal. Adult leeches were flattened by pulling the lateral edges to the sides with small pins (1 pair/segment).

Preparations were illuminated from below using fiberglass optics. Video clips (30 Hz) were taken with a CCD Camera in either color (DC 330) or black and white (Model 200T-RC or IR-1000; Dage MTV, Michigan City, IN). Images were stored on a videocassette recorder. Video clips (30 Hz) were taken with a CCD Camera in either color (DC 330) or black and white (Model 200T-RC or IR-1000; Dage MTV, Michigan City, IN). Images were stored on a videocassette recorder.

A series of video clips were taken from each animal, capturing 3–6 neighboring segments at a time for 2–10 min (Fig. 3). Care was taken to have overlapping sets of segments to assess the intersegmental phase differences between the heart constrictions along the entire length of the leech body with respect to one reference segment. We used the Imaging Workbench software (v. 4.0; Axon Instruments, Foster City, CA) for an automated analysis of heart diastole and systole. Light intensity (I) transmitted through the preparation in user-defined analysis windows drawn around corresponding sections of the lateral heart tube in each segment visible in a movie clip (see image at 1,190 ms of Fig. 3) was digitized and collected. The rhythmic filling and emptying of the heart with red blood caused oscillatory changes in the intensity of the transmitted light in the analysis windows (Fig. 3, bottom), and these “optical signals” were used in our analysis of heartbeat. The absolute values of the digitized signals depended on the size of the analysis windows and the visibility of the heart tube in a given segment and were therefore not comparable between different preparations or even between different heart segments of the same preparation.

Data series of these optical signals were imported into Kaleidagraph (Synergy Software, Reading, PA) for plotting and into Matlab (Mathworks, Natick, MA) for data analysis. First, we identified the part of the waveform representing emptying of the heart, i.e., systole, by comparing the optical signals with simultaneously acquired frame-by-frame measurements of the changes in heart tube diameter (Fig. 4). Next, we defined the start of systole (constriction) of the heart as the phase marker. In the optical data series, the start of systole was estimated best by the moment in time halfway between maximum filling of the heart segment (trough of the optical signal, Fig. 4, maximal diastole), and the moment in time of the maximal growth of the optical signal corresponding to emptying, i.e., where the first derivative (dI/dt) of the intensity trace was a maximum (d²I/dt² = 0; Fig. 4). The analysis program written in Matlab was then used to calculate the cycle period of the heartbeat and the intersegmental phase difference between a given heart segment and a reference heart segment. The cycle period of segment i was defined as the interval (Tj) between occurrences of the phase markers (i.e., start of systole) in two consecutive heartbeat cycles of the optical signal obtained from segment i. The phase (φj) of a given heart segment i was determined on a cycle-by-cycle basis using segment j as a reference (φj – φi). The phase of heart segment i was defined as the difference between the time of occurrence of the phase marker of segment i (tj) and the time of occurrence of the phase marker of the reference segment j (ti) in the same cycle divided by the reference cycle period (Tj). Phase is expressed in percent by φj − φi. A phase of 50% indicates an antiphasic or out-of-phase relationship. A positive phase difference indicates that the constriction of the reference segment leads, whereas a negative phase difference indicates it lags the constriction of a given heart segment.
All intersegmental phase differences were referenced to the heart segment 10 in a multi-step process. First, a “local” reference heart segment was chosen in each individual video clip, for example, segment 8 in a video clip of segments 5–8. The next video clip of segments 8–11 allowed us to establish the phase difference between the hearts in segments 8 and 10, thereby concatenating the data obtained from the two video clips. Side-to-side phase differences were determined for the local reference heart segment within each video clip.

Simultaneous extracellular recordings from the heart motor neuron and imaging of the heart constrictions

Innervated, reduced preparations were used to record en passant from a heart nerve while imaging the constriction pattern of the corresponding lateral heart segment. Leeches were opened dorsally and covered with leech saline [which contained (in mM) 115 NaCl, 4 KCl, 1.8 CaCl₂, 10 glucose, and 10 HEPES buffer and was adjusted to pH 7.4 with NaOH or HCl]. In such preparations, the dorsal vessel (a major collecting vessel) and much of the capillary beds are cut and blood flow is compromised. To expose the lateral heart in two segments, the crop wall and the dorsoventral muscles were removed, and the dorsal branch of the posterior nerve root was cut in these segments. The rest of the peripheral innervation was left intact. The animal was superfused with leech saline for the duration of the experiment at 3–4 ml/min. Bath volume was ~30 ml.

Of the three nerves supplying the lateral heart, the Y nerve (Wenning et al. 2004) requires the least damaging preparation and was therefore used for en passant recordings. For extracellular recordings, the Y nerve was isolated from its accompanying dorsoventral muscle fibers, and its distal part was aspirated in a small loop into a suction electrode made of a tapered glass capillary (1 mm OD) filled with leech saline. The protocol for extracellular recordings is described in detail in Wenning et al. (2004). To determine the coordination mode of the heart while imaging, two Y nerves were recorded simultaneously, which enabled us to assess the intersegmental phase difference between the heart motor neurons. Video clips (30 Hz) were taken with a CCD Camera (Model DC 330) trained in succession to the corresponding heart sections of each of the same two segments used for electrophysiological recordings.

To synchronize the acquisition of electrophysiological data with image data, we employed two different methods. In the first method, we used pClamp protocols (Axon Instruments) to trigger data acquisition by the Axon Imaging Workbench software. However, due to the long transfer time that the program needed to clear the memory buffer, data acquisition at full video rate was limited to 30 ms. We used the second method to obtain longer recordings. We triggered a short (50 ms) light pulse and simultaneously acquired the trigger signal with the electrophysiological data and the light pulse with the video camera and cassette recorder for later synchronizing. Image data were later digitized using Imaging Workbench software. To assess the constriction pattern relative to the bursts of action potentials of the corresponding heart motor neuron, the heart diameter in a given segment was measured frame-by-frame directly from the video screen. During a burst of action potentials (i.e., the burst phase), a measurement was taken from every 10th frame (interval: 330 ms) and between bursts of action potentials from every 20th frame (interval: 660 ms). For each experiment, two to four heartbeats per coordination mode were analyzed. For comparison among preparations, the heart diameter was normalized by the first five measurements after the first spike of the heart motor neuron burst. This average corresponded to the end-diastolic diameter in the first heartbeat cycle of a given preparation and was set to 100%.

The intersegmental phase differences between the heart motor neuron bursts and the instantaneous spike frequencies were calculated using a spike train analysis program written in Matlab. The analysis is described in detail in the accompanying paper (Wenning et al. 2004).

Simultaneous extracellular recordings from the vascular nerves and the heart modulatory neurons

Denervated nerve cords were used to record the activity from heart modulatory neurons in the peripheral nerves. The preparation and the recording techniques are described in the following paper (Wenning et al. 2004). In brief, leeches were opened dorsally and the vascular nerves as well as the Y nerve were exposed in four to six segments. All other peripheral nerves were cut. The head and tail brains were left attached to the ganglion chain, and the preparation was superfused with leech saline at 3–4 ml/min.

To determine the extent of innervation of the hearts by the heart modulatory neurons that are present in segments 5 and 6, we used paired extracellular recordings from one heart modulatory neuron cell body and a heart nerve. To expose the somata of a heart modulatory neuron, the segmental ganglion was freed from the surrounding ventral vessel, the segmental nerve roots cut, and the ganglion flipped to gain access to the ventral surface. The anterior lateral packet was desheathed and a suction electrode placed near the putative heart modulatory neuron soma. Gentle suction brought the cell body inside the electrode. The heart modulatory neuron was identified by size, location, and its activity pattern. Positive identification of a peripheral spike of the heart modulatory neuron required the occurrence of a spike at a fixed delay in one of the heart nerves in different segments. The fixed delay was determined through the analysis of averaged electrical activity in a time window triggered by the spikes in the soma. Total recording time was between 60 and 120 min.

Simultaneous recordings of intravascular pressure and heart modulatory neuron activity

Minimally dissected preparations were used to record intravascular pressure and heart modulatory neuron activity simultaneously. To minimize longitudinal movements, adult leeches were pinned in a stretched position (dorsal side up) in artificial pond water for a period of 30–120 min after which they were stretched maximally. Prior to dissection, the artificial pond water was replaced with leech saline, and the animal was repositioned (ventral side up).

To expose the heart in segment 8 on the right body side, a lateral incision was made from segment 7 (last annulus) to segment 9 (middle annulus). Integument and muscle layers were forced and held apart with small hooks. Care was taken to leave the protective connective tissue around the lateral heart and the dorsoventral muscles intact. To gain access to the appropriate heart accessory neuron in segment 6, a second incision was made from segment 5 (last annulus) to segment 6 (last annulus) on the ventral midline. Integument and muscle layers were forced and held apart with small hooks. The bulging mass of sex organs was kept in place by four to six smaller hooks. An incision in the ventral vessel exposed the segmental ganglion. A platform (flattened and blackened insect pin) was slipped under the ganglion and secured with dental wax at the side of the dissection dish.

Intravascular pressure was monitored using a glass capillary (1.5 mm OD) with a tip of 50–70 μm OD inserted into the lateral heart (Fig. 2). The recording pipette was connected to a precalibrated, disposable pressure transducer (DPT, Ohmeda; courtesy of Becton & Dickinson, Ventura, CA) fixed at the height of the recording site. To maximize the pressure signal, all connections between the recording site and the transducer were rigid. The transducer had a volume displacement of <1 × 10⁻³ ml/mmHg. From a reservoir, the entire system was filled with leech saline that had been filtered (Nalgene PES 0.45 μm) and degassed (using negative pressure for 2 h). To visualize developing air bubbles, the saline was colored prior to filtering with Fast Green (Sigma Chemicals, St. Louis, MO). A three-way stopcock allowed connecting the transducer to the reservoir (for filling the system), venting the transducer to air (for zeroing), and connecting the transducer to the recording probe (Fig. 2). After the
Intravascular Pressure Recording from the Heart

1. Filling
Fluid Reservoir (de-gassed leech saline)

2. Zeroing
Pressure Transducer
Open to Air

3. Recording
Recording Pipette

Ventral View

anterior sucker

posterior sucker

Heart

Statistics and nomenclature

Values are expressed as means of the averages of individual experiments ± SD. A Student’s t-test (paired or unpaired, see text) assessed statistical significance.

RESULTS

To monitor the pattern of diastole and systole along the hearts of intact, restrained animals, we devised a noninvasive imaging technique and appropriate analysis software. The weak pigmentation of juvenile leeches provided good contrast for the flow of red blood, whereas adult leeches, which are not as translucent, gave weaker signals.

The image series of Fig. 3 shows midbody segments 8–10 in an intact juvenile leech (see also the movie). In the first image (0 ms), the right hearts of segments 8 and 9 (bottom) are filled with blood, followed by filling of the heart in segment 10 (170 ms). Emptying also occurs from front to back, starting with segment 8 (340 ms) followed by the hearts in segment 9 (510 ms) and 10 (680 ms). In the contralateral left heart (top), filling starts from the rear with segment 10 (510 ms) followed by segments 9 (680 ms) and 8 (850 ms), when all visible left heart segments are filled. The heart in segment 10 empties first (1,020 ms), followed by segment 9 (1,190 ms). The different sequential orders in which the two hearts undergo diastole and systole illustrate the two coordination modes of leech hearts: the left heart with rear-to-front flow is in the peristaltic coordination mode while the right heart with seemingly front-to-rear flow is in the synchronous coordination mode (Thompson and Stent 1976a). The image series also shows that the hearts in these midbody segments beat in antiphase: while one side is in diastole and fills, the other side is in systole and empties.

For an automated analysis of the progression of systole along the hearts, analysis windows, shown in the last image (1,190 ms; Fig. 3), were placed around corresponding heart sections. Rhythmic emptying and filling of the hearts caused rhythmic light-intensity changes. This oscillatory optical signal was digitized and correlated with simultaneous diameter measurements of the hearts. The trough of the optical signal (maximal light absorption) corresponded to the maximal diastole (Fig. 4; ○) and the fast decrease of light absorption after filling of the heart corresponded to emptying (systole), which coincided with the decrease in heart diameter, i.e., constriction. We determined the intersegmental phase differences between all segments for both hearts along the entire length of the animal. We chose a phase marker equivalent to the attainment of systole (ρ; Fig. 4), and used it to calculate the cycle period and the phase differences between heart segments (see METHODS for details). The exact alignment varied somewhat because it depended on the identification of the point of end-diastolic filling, i.e., the trough of the optical signal, which was variable.

Intersegmental coordination of systole in the peristaltic and synchronous coordination modes

We calculated the intersegmental phase differences for the attainment of systole (referred to as systole hereafter) for heart segments 3–18 in 17 intact leeches (8 juveniles, 9 adults). The average heartbeat period was 4.7 ± 0.7 s in the juvenile leeches, ranging from 2.9 to 6.9 s, and 10.0 ± 3.5 s in the adults, ranging from 5.8 to 15.1 s.
Figure 5 shows the regular oscillatory optical signals associated with each heartbeat for midbody segments 8–10 on both sides in an intact juvenile leech. The optical signals are from the same preparation as used for the image series (Fig. 3), and the shaded area in the expanded optical signals on the left corresponds to the video frames shown in Fig. 3. Phase differences are referenced to the heart in segment 10, which is assigned 0%. A positive phase difference indicates that the constriction of the reference segment leads, whereas a negative phase difference indicates it lags the constriction of a given heart segment.

On average, the systole in segment 9 (L,9) in the left heart, which was in the peristaltic coordination mode, lagged by 7 ± 3% with respect to reference segment 10 (L,10), and the systole in segment 8 (L,8) lagged by 16 ± 4% (Fig. 5; top left). Systole of segment 9 (R,9) in the right heart, which was in the synchronous coordination mode, was in phase with segment 10 (phase difference: ±1 ± 4%), while segment 8 (R,8) led by

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**FIG. 3.** Consecutive images of midbody segments 8 and 10 to show the blood flow through the lateral hearts (ventral view, anterior to the left) in an intact juvenile leech. The right heart (R, bottom) is in the synchronous coordination mode, the left heart (top, L) in the peristaltic coordination mode. Heart sections filled with blood. Data from light intensity changes were collected from analysis windows from a corresponding area of the lateral hearts in each segment ( . . . at 1,190 ms) and digitized to give a numerical optical signal. The heartbeat period was 6.2 s. See also movie.

**FIG. 4.** Diameter changes of the heart tubes in segment 10 (top) correspond to changes in the optical signal captured from the same area (bottom): shown for both coordination modes. The largest diameter of the heart (maximal diastole, 100%) coincides with the trough of the optical signal (minimum light intensity, ○). Attainment of systole (◻) corresponds to the start of the decrease in heart diameter. Data are from an intact juvenile leech.
7 ± 2%, i.e., constricted earlier. The heart in segment 10 attained systole first on the peristaltic side [solid vertical line in the expanded optical signals (left)] and could thus contribute to the filling of the hearts in segments 9 and 8, which were still in diastole. Conversely, in the synchronous coordination mode, heart segment 8 constricted first and could thus deliver blood into heart segments 9 and 10, which had not entered systole yet. Front-to-rear flow appeared to be restricted, however, because the time available for filling was short due to the small phase differences on the synchronous side.

Switching between coordination modes was always reciprocal (Fig. 5; movie). Switch period and the number of beats between switches varied little in a given preparation but were quite variable across preparations (Table 1).

In contrast to the regular constriction pattern seen in midbody segments, the pattern in the front and rear segments is more complicated and more variable (Fig. 6). In anterior segments 3 and 4, the hearts spend more time in full diastole, and systole is brief when compared with segments 5 and 6, which stay constricted longer (Fig. 6, left). On the synchronous side, systole occurs almost simultaneously in segments 3–6. In the front and middle segments, phase differences between successive segments progress smoothly but are more erratic in the rear (Fig. 6, right). For example, heart segment 18 constricts later than heart segment 16 even in the peristaltic coordination mode. For comparison, we imaged adult leeches and found that blood flow was always regular in the anterior segments but often irregular from segment 12 rearward. Sometimes we observed apparent arrests of heartbeat lasting for several cycles.

Across preparations, intersegmental phase differences did not vary with period. Juvenile leeches had shorter heartbeat periods than adults, yet phase relations were similar (Fig. 7A). To further emphasize this similarity between juvenile and adult leeches, we plotted their unilateral average intersegmental phase differences separately (Fig. 7B). The phase diagram shows the systole and the diastole in each heart segment, referenced to the systole in the heart of segment 10. There was a smooth rear-to-front progression of systole in the peristaltic coordination mode. In both juveniles and adults, intersegmental phase difference between segments 14 and 4 traversed 55% (5.5%/segment), while rear segments 15–17 constricted nearly

### TABLE 1. Switch period and number of beats between switches in juvenile intact leeches

<table>
<thead>
<tr>
<th>No. of Switch Cycles per Preparation</th>
<th>Switch Period, s</th>
<th>Coefficient of Variation</th>
<th>No. of Beats per Switch Period Rounded to the Nearest Integer</th>
<th>Coefficient of Variation</th>
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<tr>
<td>10</td>
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<td>0.12</td>
<td>18 ± 2</td>
<td>0.11</td>
</tr>
<tr>
<td>7</td>
<td>86 ± 8</td>
<td>0.09</td>
<td>20 ± 2</td>
<td>0.10</td>
</tr>
<tr>
<td>8</td>
<td>43 ± 7</td>
<td>0.16</td>
<td>13 ± 2</td>
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</tr>
<tr>
<td>7</td>
<td>84 ± 11</td>
<td>0.13</td>
<td>18 ± 3</td>
<td>0.17</td>
</tr>
<tr>
<td>12</td>
<td>96 ± 10</td>
<td>0.10</td>
<td>20 ± 2</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Values are means ± SD.

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*Fig. 5.* Intersegmental phase differences in systole in middle segments 8–10 on the left ([L,8–L,10; top 3 traces] and right ([R,8–R,10; bottom 3 traces] in an intact juvenile leech across a switch in coordination mode. The expanded trace on the left emphasizes the differences between the 2 coordination modes. The shaded box corresponds to the same heartbeat cycle as shown in Fig. 3. Diamonds indicate the systole for each heartbeat cycle connected by dotted lines for visual guidance. The solid vertical line in the expanded optical signals (left) indicates the time that the first heart segment attains systole (segment 10 on the peristaltic side, segment 8 on the synchronous side). Switching occurred during the 3rd cycle and was reciprocal. The average phase difference is indicated for each segment and is referenced to ipsilateral heart segment 10 (0% phase; boxed diamonds).
Simultaneously, there was a front-to-rear progression of systole in the synchronous heart between segments 3 and 13 traversing \( \approx 20\% \) of phase (2%/segment). Phase progression was smoother in the juveniles than in the adults where phase differences were generally more variable. In the rear, phase relations were more variable in both juvenile and adult leeches.

**Side-to-side coordination of systole in intact leeches**

To understand blood flow in the intact leech, we need to know the coordination between the two hearts. The bilateral phase diagrams of Fig. 8 summarize the side-to-side phase differences for juveniles (left) and adults (right). Systole in the heart of segment 10 on the peristaltic side is assigned 0% phase (---). The phase difference between the peristaltic and the synchronous heart in segment 10 was similar in juvenile and adult leeches (+60.2 \( \pm \) 11.4\%, 8 juveniles; +62.1 \( \pm \) 5.1\%, 9 adults), and the systole in the heart segment 10 on the synchronous side was shifted accordingly to the right. To illustrate the coordination in the rear segments, the peristaltic side is duplicated and shifted to the right by 100% (---).

The hearts in segment 9 constricted in antiphase. In the front, heart constrictions came nearly in phase, with the peristaltic side leading the synchronous side by \( \approx 15\% \). In the rear, heart constrictions also came nearly in phase up to segment 15, with the synchronous side leading by \( \approx 15\% - 20\% \). Rear segments 16–18 constricted almost simultaneously (Fig. 8).

The rear-to-front progression of systole in the peristaltic coordination mode supports rear-to-front blood flow and is in accordance with previous findings that, in addition to blood entering from the two afferent vessels, blood also enters from posterior segments (Kralh and Zerbst-Boroffka 1983) (Fig. 1). Blood flow had been thought to be rear-to-front in the hearts for both coordination modes (Boroffka and Hamp 1969), but the slight front-to-rear progression of systole in the synchronous coordination mode shown here and the visual observations of blood flow in our video clips do not support forward blood flow (Figs. 3, 6, and 7).

**Correlation of heart motor neuron activity and heart constrictions**

Leech hearts are innervated by 16 pairs of segmentally arranged motor neurons (Thompson and Stent 1976a), which form neuromuscular junctions on the heart muscle cells (Maranto and Calabrese 1984a). As shown by intracellular recordings from both the heart muscle and its innervating motor neuron, excitatory junctional potentials from the heart motor neurons summate in the heart muscle cells and give rise to plateau-like potentials (Maranto and Calabrese 1984b). The heart motor neurons reset and entrain the heart’s intrinsic electrical and contractile myogenic rhythms (Maranto and Calabrese 1984b).

We wanted to align the systole in a specific heart segment with the electrical activity of the corresponding heart motor neuron to later combine the constriction pattern described here with the fictive motor pattern described in the following paper (Wenning et al. 2004). We therefore monitored heart motor neuron discharge and the constriction of the corresponding heart segment using simultaneous video monitoring and en passant recordings and determined the phase difference between heart constriction and heart motor neuron activity (Fig. 9, cartoon, and METHODS). To visualize the hearts and have access to the heart nerves, we used reduced preparations in which the innervation was left intact but circulation was disrupted and focused on segments 4, 7, 10, and 14. We chose the Y nerve for en passant recording of the heart motor neuron discharge because it requires the least dissection (see Wenning et al. 2004 for a description of the heart innervation). In these preparations, the automated analysis of heart constriction described in the preceding text for intact leeches did not yield reliable optical signals. Instead, we measured the heart diam-

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**FIG. 6.** Examples of the intersegmental phase differences in systole (diamonds) for front segments 3–6 (left) and rear segments 12–18 (right) from 2 intact juvenile leeches. In the front segments, systole progresses rear-to-front on the peristaltic side and occurs almost simultaneously on the synchronous side (left), while the rear segments constrict nearly simultaneously in both coordination modes (right). Note that systole is brief in front segments 3 and 4. Mean phase differences are referenced to segment 6 (front) and to segment 16 (rear).
eter frame by frame. For each preparation, we referenced the diameter changes of the heart tube to the end-diastolic diameter of its first cycle and set this value to 100%. On average, the lateral hearts constricted to between 70 and 40% of their end-diastolic diameter. Here, systole was defined as a drop by 20% of the full constriction in that heart segment and corresponded well to the systole defined in the analysis of the optical signals in intact animals (Fig. 4). We referenced the systole to the median spike of the corresponding heart motor neuron burst (see Wenning et al. 2004) for a detailed description of the extracellular recordings from the heart motor neurons.

The example shown for segment 14 illustrates the timing of heart constriction with respect to the median spike in the corresponding heart motor neuron during a switch in coordination mode (Fig. 9). Constrictions are of the same magnitude in both coordination modes, albeit shorter during the switch. Figure 10 shows that timing of heart systole with respect to motor neuron discharge was not uniform along the body axis. In the more anterior segments 4 and 7, the hearts constricted around the median spike of the corresponding motor neuron burst and the constriction outlasted the heart motor neuron burst. In the more posterior segments 10 and 14 (Figs. 9 and 10) as well as 17 and 18 (data not shown), heart constrictions started before the median spike and hearts dilated while the heart motor neuron was still active. Furthermore, constrictions in the front segments started at higher frequencies of the heart motor neuron discharge than constrictions in the rear. The timing of, and the change in, heart diameter were segment-specific regardless of coordination mode. The heart diameter changed most in the rear segments to ~40% of its end-diastolic diameter (Fig. 10). We conclude from these experiments that the hearts constrict in a fixed phase relation with respect to their corresponding heart motor neuron bursts.

Action of the heart modulatory neurons on blood pressure

Two pairs of heart modulatory neurons, present in segmental ganglia 5 and 6, innervate the contralateral heart sections of segments 5 and anterior and segments 6 and posterior, respectively (Calabrese and Maranto 1984; Maranto and Calabrese 1984b). We used paired extracellular recordings from the heart nerves and the corresponding heart modulatory neuron to re-examine their contribution to heart innervation with special attention to posterior segments in denervated nerve cords (Fig. 11A). Action potentials from the heart modulatory neuron in segment 6 were recorded with a fixed delay in the heart nerves up to segment 12 (Fig. 11B) but not in segments 13 (n = 2), 14 (n = 2), and 16 (n = 1). Action potentials recorded in the soma of heart modulatory neurons in segment 5 were also recorded in the heart nerves of segments 5, 4 (data not shown), and 3 (Fig. 11C), confirming earlier results (Calabrese and Maranto 1984b).
Thus the heart modulatory neuron in segment 6 extends into the heart nerves up to segment 12, while the heart modulatory neuron in segment 5 extends into the heart nerves of segments 3–5.

Occasionally, extracellularly recorded action potentials were larger than those of the heart motor neurons and could be reliably identified on an outgoing nerve (Fig. 11D, top,*). We use this example to show the different bursting patterns of the heart modulatory neurons in segments 5 and 6 in both coordination modes in denervated nerve cords. In the heart modulatory neuron of segment 5, action potentials were not grouped in clear bursts (Fig. 11D, top,*). On average, the heart modulatory neurons in segment 5 had an irregular, rather tonic firing pattern. The heart modulatory neurons of segment 6 had regular bursts and mean spike frequencies were on average lower (0.69 ± 0.46 Hz) in the synchronous coordination mode than in the peristaltic coordination mode (0.85 ± 0.40 Hz; means of 7 preparations; *P < 0.02; paired t-test).

The paired recordings from heart motor and modulatory neurons enabled us to calculate the phase relations between the co-projecting heart motor neurons and the heart modulatory neuron in segment 6. The heart modulatory and motor neurons burst roughly in phase on the peristaltic side, while the burst of the heart modulatory neuron of segment 6 is out of phase with the co-projecting heart motor neurons on the synchronous side, confirming earlier results (Calabrese and Maranto 1984). The pattern of activity of the heart modulatory neurons differed in extracellular (Fig. 11D) versus intracellular recordings (Fig. 12, C and D) (Calabrese and Maranto 1984). Lower frequencies of discharge and less well-defined bursts were observed in extracellular recordings, especially in the heart modulatory neuron 5 (Fig. 11D).

The heart modulatory neurons form synapses on the heart muscle cells (Maranto and Calabrese 1984a), induce myogenic activity in quiescent hearts, and accelerate the rhythm of hearts that are already myogenic. The heart modulatory neurons can also increase the beat tension in hearts that are entrained by rhythmic discharge of the heart motor neurons driven by the heartbeat central pattern generator (Calabrese and Maranto 1984). Because the hearts display different pressure profiles in the two coordination modes (Krahl and Zerbst-Boroffka 1983) and because the heart modulatory neurons modulate the contractile strength of the hearts (Calabrese and Maranto 1984), we examined whether blood pressure is determined by the heart modulatory neurons (Fig. 12). We monitored intravascular pressure in the lateral heart of segment 8 in minimally dissected leeches, while recording intracellularly from the contralateral heart modulatory neuron in segment 6 (Fig. 12, cartoon). Systolic pressures are about twice as high in the peristaltic coordination mode as in the synchronous coordination mode (Fig. 12A). Systolic pressure was between 20 and 45 mmHg in the peristaltic coordination mode and 10 and 20 mmHg in the synchronous coordination mode. To record intracellularly from the heart modulatory neuron in the segmental ganglion, we had to open the ventral vessel. The inevitable blood loss might be responsible for somewhat lower systolic pressures than reported previously (peristaltic side: 48 ± 14 mmHg; synchronous side: 26 ± 11 mmHg) (Krahl and Zerbst-Boroffka 1983).

Depolarization of the heart modulatory neuron in segment 6 (+1 nA, 30–50 s) led to an increase of the systolic pressure both in the peristaltic coordination mode (from 21 to 36 mmHg; data not shown) and, more pronounced, in the synchronous coordination mode (from 11 to 30 mmHg) as well as to an increase of the diastolic pressure (Fig. 12B). Conversely, hyperpolarization of the heart modulatory neuron (−1 nA, 30–50 s) resulted in a decrease of the systolic pressure in the peristaltic coordination mode (Fig. 12C, 1st current injection) and across a switch from the peristaltic to the synchronous coordination mode (Fig. 12C, 2nd current injection). It took one to two cycles for the systolic pressure to change on injecting current into the heart modulatory neuron and sometimes more cycles to wane. This delayed onset and waning of the peripheral effects of the heart modulatory neuron is consistent with its modulatory role (Calabrese and Maranto 1984). Neither the heartbeat period nor the timing of the switching was affected by the depolarization or hyperpolarization of the heart modulatory neurons. We conclude from these experiments that the heart modulatory neurons may modulate, but do not determine, the difference in systolic blood pressure in the two hearts.

**DISCUSSION**

Two longitudinal vessels serve as hearts in the leech (Boroffka and Hamp 1969). Aided by sphincters and valves (Fig. 1) (Hammersen et al. 1976), they propel blood along the
body axis and distribute blood into the segmental circulation. Leech hearts are myogenic, but heart rate and the coordination of the constriction of the individual heart segments are shaped by the motor pattern, which in turn is driven by the heartbeat central pattern generator (Calabrese and Maranto 1984; Calabrese et al. 1995; Hill et al. 2003; Thompson and Stent 1976a,b). Leech hearts switch between two coordination modes termed the peristaltic coordination mode and the synchronous coordination mode, associated with different systolic pressure profiles (Krahl and Zerbst-Boroffka 1983; Thompson and Stent 1976a). Our segment-by-segment analysis of the coordination of systole shows that in the peristaltic coordination mode posterior heart segments fill and empty first and that in the synchronous coordination anterior heart segments fill and empty first (Fig. 3). Intersegmental phase differences do not vary with the heartbeat period (Fig. 7A), perhaps ensuring similar hemodynamics when the heart rate changes.

Interaction of neuronal activity and heart performance

Heart systole in the intact animal results from the interaction of the muscular heart tubes with both the heart modulatory and motor...
neurons and also with the variable load associated with blood entering and leaving the hearts. Timing of the constriction in the intact leech might additionally depend on other factors including afferent feedback, neuromuscular transform, the variable load environment, and the hydrodynamics of the vascular system.

Heart modulatory neurons increase the beat tension in hearts that are entrained by the heart motor neurons through the central pattern generator (Calabrese and Maranto 1984), suggesting a role in shaping the different systolic pressure profiles associated with the two coordination modes. As shown here, identified heart modulatory neurons do not project to heart segments more posterior than segment 12 but systolic pressures still differ as far back as segment 14 (Krahl and Zerbst-Boroffka 1983). The heart modulatory neurons may modulate blood pressure when their pattern of activity changes (Fig. 12, B and C). However, they do not determine the different systolic pressure profiles characteristic for the two coordination modes. Rather, these strikingly different systolic pressures seem to be generated by the intersegmental coordination of systole and hence by the rhythmic activity pattern of the heart motor neurons (see following text).

Each heart motor neuron burst caused a constriction in the corresponding heart segment but with segment-specific phase differences both with respect to onset and waning of the systole (Figs. 9 and 10). In principle, these differences may be due to differences in motor neuron discharge (spike frequency, duty cycle) at the neuromuscular junction, of the contractile properties of the heart muscle cells, and/or of the hemodynamic load and vessel mechanics in intact versus reduced preparations. For example, in intact animals, filling of the hearts dilates the heart tube, which might delay its constriction. Conversely, because there are no antagonistic muscles, rapid dispersion in leech hearts may rely on incoming blood. Expansion of the hearts during diastolic filling is limited by the elasticity of the heart and potentially by hydro skeletal constraints. To resolve this issue, we need to study the neural control of the coordination of the hearts and the contractile incoming vessels and heart performance in minimally dissected animals.

Switching between coordination modes is under neural control (Calabrese 1977). Whether considering the fictive motor pattern (Calabrese and Peterson 1983; Lu et al. 1999; Thompson and Stent 1976a; Wenning et al. 2004), the motor pattern...
in minimally dissected leeches (Krahl and Zerbst-Boroffka 1983) or intact leeches (this study), switching between the two coordination modes is always reciprocal. Switch period and the number of beats between switches vary little in a given preparation but are quite variable across preparations (Table 2 in Wenning et al. 2004).

**Circulation**

The segment-by-segment analysis of the pattern of systole in the peristaltic and the synchronous hearts results in an improved understanding of circulation in the leech. Most importantly, we found that blood flow is not always forward in the hearts as previously thought (Boroffka and Hamp 1969).

Sphincters and valves are present in the contractile vessels and aid in directing blood flow. The hearts have a main sphincter in the posterior part of each segment (Fig. 1). The main sphincter appears as a thickening of the heart musculature wall (Maranto and Calabrese 1984a) and obstructs blood flow early in systole. The initial portion of the single segmental outlet, the lateroabdominal vessel, is muscular and may be closed off during systole (Hildebrandt 1988). The orifices of the two afferent vessels bear multiple pine cone-shaped fibrous villi, which surround the opening (Hammersen et al. 1976). Nonmuscular in nature, they operate as (passive) valves (Fig. 1). When systolic pressure in the heart overcomes the pressure in the afferent vessels, the villi are pushed back into the orifice preventing backflow (Fig. 1, emptying in systole).

In the peristaltic coordination mode, nearly simultaneous contractions in segments 17–14 act as a resistance to backflow that is followed by a peristaltic contraction wave propelling the blood forward (Figs. 7 and 8). Filling of the hearts in segment 13 and anterior is through the two afferent vessels and from the rear (Fig. 1). The lateroabdominal sphincter is open for the initial phase of the contraction cycle in each segment, and therefore some blood may enter the segmental circulation (Hildebrandt 1988). The valves to the afferent vessels close early in systole, preventing flow back into the periphery. At the same time, the sphincters located in the posterior portion of the hearts close and prevent backflow during the rear-to-front peristaltic wave. The peristaltic heart pumps blood into the vessels of the front sucker region perfusing the associated capillary beds and causing rearward flow into the dorsal and ventral vessels and the synchronous heart. We hypothesize that the peristaltic heart is the main propulsive force to drive blood along the body axis to the front as well as back to the rear.
FIG. 12. Intravascular pressure recordings in a semi-intact preparation from the heart in segment 8 on the right body side [Heart(R,8)] while simultaneously recording intracellularly from the heart modulatory neuron in segment 6 on the contralateral side [HA(L,6); cartoon, right]. A: high systolic pressures are found in the peristaltic coordination mode and low systolic pressures in the synchronous coordination mode. B: depolarization of the heart modulatory neuron causes an increase in systolic and diastolic blood pressure in the synchronous coordination mode. C: hyperpolarization of the heart modulatory neuron causes a decrease in the systolic pressure in both coordination modes. *, the switches between coordination modes.
Rearward blood flow into the synchronous heart through interconnecting vessels in the front sucker region is limited because systole begins soon after the end of the peristaltic wave in the opposite heart (Fig. 8). The synchronous heart, however, does not contribute to flow into the anterior capillary beds or into the dorsal and ventral vessels as had been thought previously (Boroffka and Hamp 1969). Rather, as suggested by Hildebrandt (1988), the synchronous heart may support segmental circulation through the lateroabdominal vessels in each segment. However, our results do not support propulsive rearward flow. Systole progresses front-to-rear but phase differences from segment to segment are much smaller than on the peristaltic side (Fig. 7), and it is difficult to envision how such a small contraction progression can support propulsive rearward blood flow. Moreover, near synchrony in segmental contraction should support peripheral flow by restricting both forward and rearward flow: the main splanchners along the synchronous heart close in rapid succession, the valves to the afferent vessels close, and blood, trapped in one heart segment, may be forced out mainly through the lateroabdominal vessel into the peripheral circulation (Fig. 1). The absence of a distinct pressure pulse, which would signal a propulsive force from an anterior segment, and the lower systolic pressures in the synchronous heart (Figs. 1 and 12D), would seem to support this view. However, we cannot predict at this point how much of the blood entering a given segment in the synchronous heart ends up in the peripheral circulation of that segment or flows into more posterior segments. Neither video imaging as used in this study nor the two-point pressure recordings from various locations employed previously (Hildebrandt 1988; Kralh and Zerbst-Boroffka 1983) are suitable to track a defined bolus of blood.

In segmented animals with a closed circulatory system like annelids, circulation serves serial and parallel vascular beds at the same time. The demands on circulation—rapid blood transfer to deliver nutrients and oxygen along the body axis and slower circulation along the segmental capillary beds—are met differently among annelids. The design, albeit not the evolutionary origin, of the annelid vasculature is deceptively similar, yet, the physiological role of these vessels differs and essentially among annelids. The design, albeit not the evolutionary origin, of the annelid vasculature is deceptively similar, but differences from segment to segment are much smaller than on the peristaltic side (Fig. 7), and it is difficult to envision how such a small contraction progression can support propulsive rearward blood flow. Moreover, near synchrony in segmental contraction should support peripheral flow by restricting both forward and rearward flow: the main splanchners along the synchronous heart close in rapid succession, the valves to the afferent vessels close, and blood, trapped in one heart segment, may be forced out mainly through the lateroabdominal vessel into the peripheral circulation (Fig. 1). The absence of a distinct pressure pulse, which would signal a propulsive force from an anterior segment, and the lower systolic pressures in the synchronous heart (Figs. 1 and 12D), would seem to support this view. However, we cannot predict at this point how much of the blood entering a given segment in the synchronous heart ends up in the peripheral circulation of that segment or flows into more posterior segments. Neither video imaging as used in this study nor the two-point pressure recordings from various locations employed previously (Hildebrandt 1988; Kralh and Zerbst-Boroffka 1983) are suitable to track a defined bolus of blood.

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