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Assessment of the pressure–volume relationship of the single ventricle of the grass shrimp, *Palaemonetes pugio*

J. A. Guadagnoli[1,2,*], K. Tobita[3] and C. L. Reiber[2]

[1]*College of Osteopathic Medicine, Touro University – Nevada, Henderson, NV 89014, USA,* [2]*Department of Biological Sciences, University of Nevada, Las Vegas, NV, USA and* [3]*Department of Pediatrics, Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA, USA*

*Author for correspondence (e-mail: jguadagnoli@touro.edu)

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Summary

The ventricular pressure–volume (PV) relationship has been used extensively to study the mechanics and energetics in multi-chambered hearts of closed circulatory system vertebrates. In the current study we applied the use of PV loops in the assessment of cardiac mechanics and energetics in the single ventricle of a decapod crustacean possessing an open circulatory system. Anatomical differences between multi- and single-chambered hearts include multiple ostia entering and valved multiple arterial systems exiting the ventricle, and the neurogenic origin of the heartbeat in decapod crustaceans. However, the microscopic architecture and excitation–contraction coupling events are similar in both systems. Ventricular pressure and area were obtained independently and integrated into pressure–area loops. Area was then converted to volume to generate PV loops. Based on the PV loops generated in this study, the ventricle of *Palaemonetes pugio* processes the same primary phases of the cardiac cycle as ventricles from the multi-chambered hearts of vertebrates: (1) isovolumic contraction, (2) ventricular emptying, (3) isovolumic relaxation and (4) ventricular filling. The area enclosed by the PV loop provides a measure of stroke work and when multiplied by heart rate provides an assessment of cardiac work. This initial examination of PV loops from a single-ventricle decapod crustacean demonstrates the utility of this technique to further elucidate the cardiac mechanics and energetics of this system, and in particular during times of physiological stress.

Key words: invertebrate, cardiac function, stroke work, *Palaemonetes pugio*.

Introduction

The work performed by the ventricle is a function of heart rate and contractility. Contractility, the amount of force generated by the myocardium to pump blood throughout the vascular system, is reflected in stroke work. Pressure–volume (PV) loops provide a mechanism to directly access stroke work per cardiac cycle and hence myocardial O_2 consumption (Sagawa et al., 1988). More recently, pressure–area (PA) loops have been used to define cardiac dynamics in mammalian (Senzaki et al., 2001) and avian systems (Tobita and Keller, 2000). Both PV and PA loops have been used extensively to study the energetics of cardiac contraction and dynamics of cardiac function in the ventricles of mammalian and avian systems, yet this tool has found limited use in the study of cardiac dynamics in the single ventricle of animals possessing an open circulatory system (Wilkens, 1999). We believe that PV and PA loops provide a valuable tool in the assessment of the cardiovascular dynamics of the single ventricle.

The cardiovascular system can be loosely defined as a three-part system with (1) a pump for generating force to move (2) blood or hemolymph through a (3) distribution pathway or arterial system. Alterations in any of the three components can alter the amount of work required of the pump. In decapod crustaceans the pump consists of a single ventricle suspended within a pericardial sinus by a three-dimensional array of suspensory ligaments located anteriorly, laterally and posteriorly on the dorsal and ventral surface of the heart (Maynard, 1960; Blatchford, 1971). All ligaments are paired, with the exception of dorsal posterior suspensory ligament (Blatchford, 1971). Unlike the typical vertebrate four-chambered heart, with only one entrance and one exit from the ventricle, crustaceans must coordinate the opening and closing of multiple ostial and aortic valves. Pre-branchial hemolymph from active tissues is collected in large, paired infrabranchial sinuses that guide the hemolymph back to the gills to become reoxygenated. Post-branchial hemolymph enters defined branchio-cardiac veins that deliver the oxygenated hemolymph to the pericardial sinus surrounding the heart. This defined path serves to minimize admixture of oxygenated and deoxygenated hemolymph (McLaughlin, 1983). Hemolymph from the

pericardial sinus enters the heart passively through three pairs of muscular ostia (Fig. 1B,C), and leaves the heart *via* six aortic valves that lead to five arterial systems (Fig. 1A). Contraction of the ventricle then distributes the oxygenated hemolymph to the arterial systems that branch repeatedly to terminate as open tubes through which hemolymph flows to directly bathe the tissues (McLaughlin, 1983).

Physiologically, the initiation of cardiac contraction in the single ventricle of a decapod is neurogenic, as compared with the myogenic properties of vertebrate cardiac myocytes, and is driven by a burst of action potentials from the cardiac ganglion located on the inner dorsal surface of the heart (Florey, 1960; Sullivan and Miller, 1984). Overall cardiac function depends on ganglionic burst frequency and duration (for a review, see Cooke, 2002), which is further altered by both cardio-excitatory and cardio-inhibitory nerves originating from the central nervous system. Beyond the difference in the initiation of contraction, the microscopic architecture of cardiac myocytes of crustaceans is similar to that of typical mammalian myocytes, with each sarcomere spanning the area between two Z-lines and surrounded by the sarcoplasmic reticulum (SR) and T-tubule system (Nylund et al., 1987). The SR membrane system is involved in excitation–contraction (EC) coupling (Yazawa et al., 1999; Shinozaki et al., 2002) with activation of voltage-dependent sarcolemmal Ca^{2+}-release channels that allow the entry of Ca^{2+} required to initiate contraction. As in all muscle cells, relaxation occurs when released Ca^{2+} is sequestered back into the SR or pumped out to the extracellular fluid, an energetically demanding process. From the available literature, the microscopic architecture of the contractile apparatus of crustacean myocardium shares many similarities with that of mammalian myocardium (Shinozaki et al., 2002; Yazawa et al., 1999).

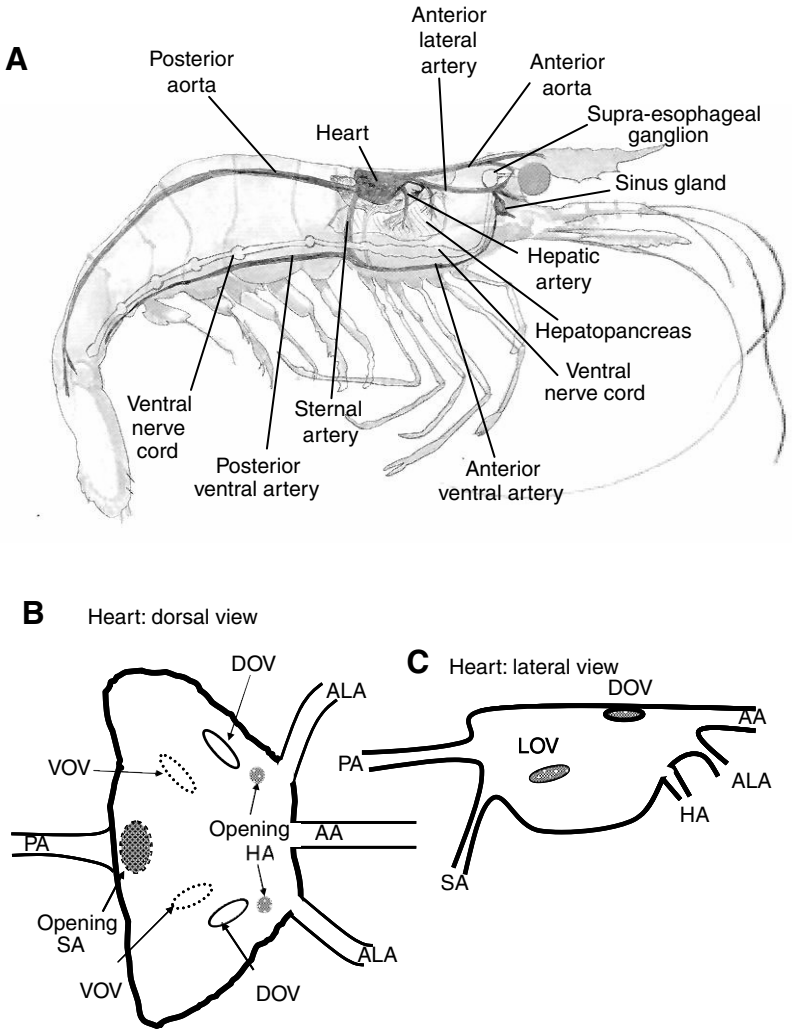

Fig. 1. (A) Overview of circulatory anatomy of a shrimp. (B) Dorsal view of heart. (C) Lateral view of heart. AA, anterior aorta; ALA, anterior lateral artery; DOV, dorsal ostial valve; HA, hepatic artery; LOV, lateral ostial valve; PA, posterior aorta; SA, sternal artery; VOV, ventral ostial valve.

The PV loop (Fig. 2) provides a tool for the estimation of myocardial O_2 consumption. There are four distinct phases that include (1) isovolumic contraction as pressure is generated by the ventricle, (2) ventricular emptying as the pressure in the ventricle overcomes peripheral pressure, (3) isovolumic relaxation as the ventricle relaxes and (4) rapid ventricular filling at low pressure (Berne and Levy, 1986). The *x*-axis provides an estimate of stroke volume and the *y*-axis represents changes in pressure during the cardiac cycle. Although ventricular area and pressure can be determined independently for the determination of stroke volume, or total pressure change, the integration of this data is the basis for determining myocardial O_2 consumption. Once integrated, the area enclosed by a PV loop is an index of kinetic energy or ventricular stroke work (Sagawa et al., 1988).

Given the physiological similarities between ventricles from vertebrate closed circulatory systems to those of ventricles from the open circulatory system of a decapod crustacean, we sought to generate PV and PA loops from the single ventricle of a decapod crustacean. These loops would allow for a detailed assessment of stroke work and cardiac dynamics in a single ventricle that has multiple inflow and outflow valves. We used the grass shrimp, *Palaemonetes pugio*, to test the hypothesis that PV loops from this multi-outlet single ventricle would be comparable to the PV loops generated from the ventricle of the vertebrate closed circulatory system.

Materials and methods

Animal preparation

Grass shrimp, *Palaemonetes pugio*, were purchased from Gulf Specimen Marine Laboratories, Inc. (Panacea, FL, USA), and maintained in 20 L aquaria in aerated seawater (30–32 ppt

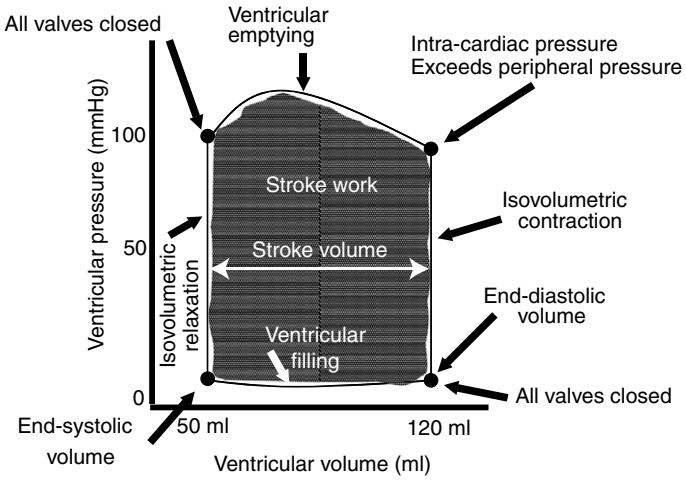

Fig. 2. Pressure–volume loop of the left ventricle for a single cardiac cycle [adapted from Berne and Levy (Berne and Levy, 1986)].

at 20°C). Animals were maintained in laboratory conditions for two weeks prior to experimental use and were fed marine flakes (Tetra) three times a week. Experimental animals were separated from the general population and fasted two days prior to use.

Grass shrimp were attached to the flattened end of a wooden applicator stick at the lateral cephalothorax with cyanoacrylate glue. The animal was held in place and positioned within the experimental chamber with a micromanipulator (World Precision Instruments, Sarasota, FL, USA). The video camera was placed over the chamber so that video images of the heart could be captured through the transparent exoskeleton [see methods from Harper and Reiber (Harper and Reiber, 1999)]. The transparent exoskeleton allows for the measurements of area and pressure *in vivo*.

Experimental design

Seawater (30±2 ppt) within a flow-through experimental chamber was maintained at 20°C and the partial pressure of oxygen (P_{O_2}) in the water was maintained at normoxic levels by bubbling room air into the flow-through chamber. All animals were placed in the experimental chamber in normoxic water (P_{O_2}=20.5 kPa) and acclimated for 1 h. Thereafter a minimum of three recordings of pressure and volume were made for each animal.

Intraventricular pressure

Intraventricular pressure was measured using a servo-null pressure system (model 900A; World Precision Instruments) and an analog–digital (AD) board (DAQPad 6020-50E; National Instruments, Austin, TX, USA) at a rate of 600 Hz. A glass micropipette with a 2–5 μm diameter tip was filled with 3 mol l^{-1} NaCl and positioned in the ventricle with the use of a micromanipulator (World Precision Instruments). The micropipette tip was inserted through the soft dorsal arthrodial membrane at the junction of the thorax and abdomen to minimize disturbance to the animal, and then slowly advanced into the ventricle. The servo-null system measures the resistance of the 3 mol l^{-1} NaCl-filled pipette tip and prevents changes in resistance by generating an opposing pressure to the pressure present at the tip. Intraventricular pressure was calculated after correcting for the 'zero-pressure' or calibration pressure, recorded when the tip was placed in the experimental chamber at a level adjacent to the heart.

Video image processing

Video images were acquired *in vivo* through the transparent exoskeleton at a rate of 60 Hz using a stereo-microscope (Leica MZ12.5; McBain Instruments, Chatsworth, CA, USA) equipped with a video camera (World Precision Instruments), frame-grabber board (LG-3; Scion, Frederick, MD, USA) and programmed frame-grabbing software (Scion Image; Scion). Each video image was analyzed using custom-programmed image analysis software (LabView; National Instruments) commonly used in the study of chick embryos (Tobita and Keller, 2000). First, maximum and minimum ventricular borders were traced from recorded sequences to determine ventricular cross-sectional area. The number of pixels and individual pixel values in the area contained between the maximum and minimum borders was stored in memory as a region of interest (ROI) (Fig. 3). Assuming that movement of the ventricular border would be associated with changes in the pixel values within the image of the heart, changes in

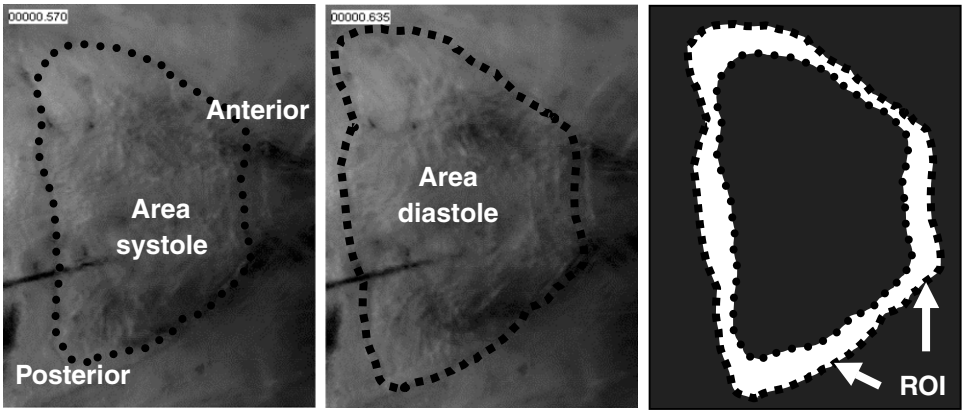

Fig. 3. Dorsal view of the heart through the carapace. (A) Outline of heart in systole defines the minimal area. (B) Outline of heart in diastole defines the maximal area. The area between the maximal and minimal area defines the ROI used in automated area analysis.

ventricular area from the minimum area during the cardiac cycle were identified automatically by detecting the pixels that changed value in the ROI for sequential video fields. Total ventricular cross-sectional area in each video field was then calculated as the sum of the changes in area within the ROI defined by the maximum (Fig. 3B) and minimum (Fig. 3A) ventricular areas. The pressure signal (600 Hz) and video images (60 Hz) were acquired simultaneously for 4 s by an output trigger to the AD board and the frame-capturing board. Using a custom computer program (K. Tobita using LabView; National Instruments) the pressure waveform was interpolated with the image data to yield a series of x, y coordinates required for the PA loop.

Area was converted to volume in a method used in previous studies (Harper and Reiber, 1999; Guadagnoli and Reiber, 2005). The use of dimensional analysis, with the heart modeled as a trapezoid {cardiac volume=w [0.5 $h(b + a)$], where w is width, h is height, a is base length and b is top length; the width (w) of the heart was determined to be 0.64 h during systole and 0.67 h during diastole}, differed only 13% from dye dilution techniques (Harper and Reiber, 1999). We therefore used the same model and by incorporating the known changes in the depth of the heart from a lateral view, converted area to volume (Harper and Reiber, 1999; Guadagnoli and Reiber, 2005). The volume data was then used to generate PV loops.

Heart rate (f_H), maximum pressure (P_{max}), minimum pressure (P_{min}), change in pressure (ΔP), maximum area (A_{max}), minimum area (A_{min}) and change in area (ΔA) were determined by independently analyzing the pressure and video output from LabView (National Instruments) using a customized computer program, MATLAB (The Mathworks, Inc., Natwick, MA, USA). Area was converted to volume to obtain end-diastolic volume (EDV), end-systolic volume (ESV) and stroke volume (V_s). After interpolation of the PV data to generate multiple loops in LabView, the data were analyzed using MATLAB to obtain a mean PV loop as well as the area enclosed by the loop. The area of the PV loop is an estimate of stroke work (SW). The PV loop does not account for heart rate; therefore, the product of area and f_H yields an estimate of minute cardiac work (CW). However, either a PV or PA loop can be used to elucidate the phases of the cardiac cycle and cardiac dynamics in general. All values are means ± s.e.m. (N=12).

Results

Independent measures of pressure and area allow for assessment of the cardiac cycle, prior to the data points being integrated to form a PA loop. Two representative cardiac cycles are shown in Fig. 4. Time between pressure peaks was used to determine f_H. Mean heart rate for six cardiac cycles (N=12 animals) was 285±3.6 beats min^{-1} with 53±0.75% of time spent in systole and 47±0.75% of time spent in diastole. The mean change in pressure was 29.4±1.2 mmHg (1 mmHg=1.333 Pa) with mean P_{max} and P_{min} values at 20.9±1.3 and –8.5±0.7 mmHg, respectively. Mean change in area was 0.048±0.008 mm^2 with an A_{max} value of 0.8011±0.063 and A_{min} of 0.7593±0.052. After conversion to volume, EDV was 572.8±14.5 μl beat^{-1} and ESV was 470.6±12.8 μl beat^{-1}, resulting in a mean V_s of 102.2±3.2 μl beat^{-1} that, when multiplied by f_H, results in a V_b value of 29.0±1.5 ml min^{-1}.

A representative ventricular pressure tracing acquired at 600 Hz (Fig. 5A) and an area tracing resulting from digital image analysis of the ROI (Fig. 5B) are plotted on a single x-y coordinate system to yield eight PA loops (Fig. 5C). The MATLAB program calculates the area enclosed by the mean loop (Fig. 5D, solid line). Mean SW calculated from the area of the mean PV loop (Fig. 6, solid line) and CW are 496.73±21.92 mmHg mm^3 and 142×10^3 mmHg mm^3 min^{-1}, respectively. The PV loop generated from the grass shrimp ventricle has the same four phases seen in Fig. 2 and will be discussed in detail.

As a demonstration of the utility of PV loops in assessing changing cardiac dynamics, we exposed a grass shrimp to hypoxia (6.8 kPa) for 30 min. In this animal, hypoxic conditions resulted in a decrease in ΔP, an increase in V_s and a decrease in PV area (SW) from 377.3 to 343.2 mmHg mm^3 (Fig. 7).

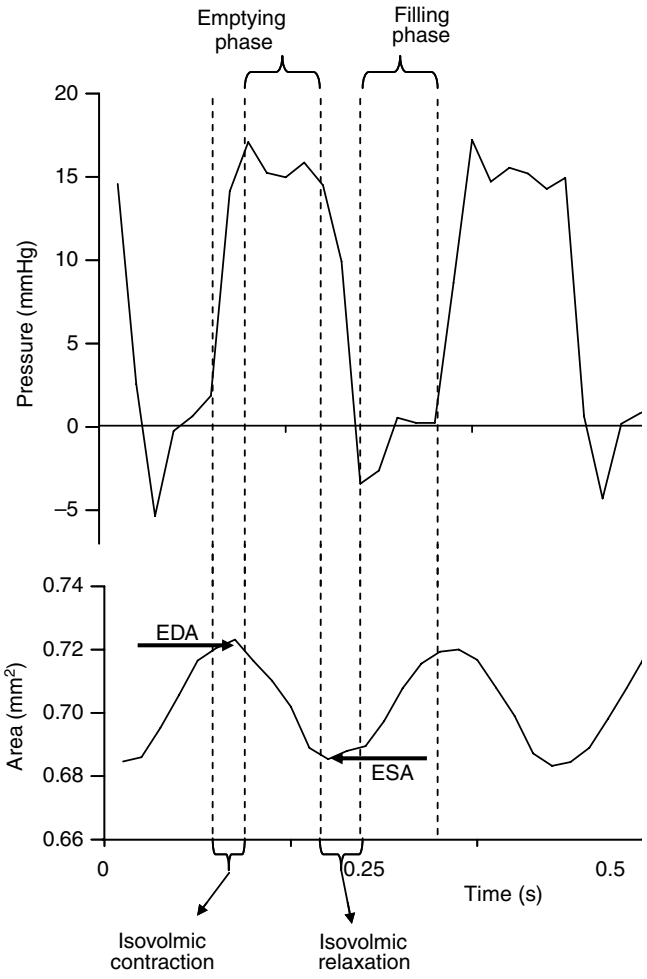

Fig. 4. Pressure and area tracing for two cardiac cycles. EDA, end-diastolic volume; ESA, end-systolic volume.

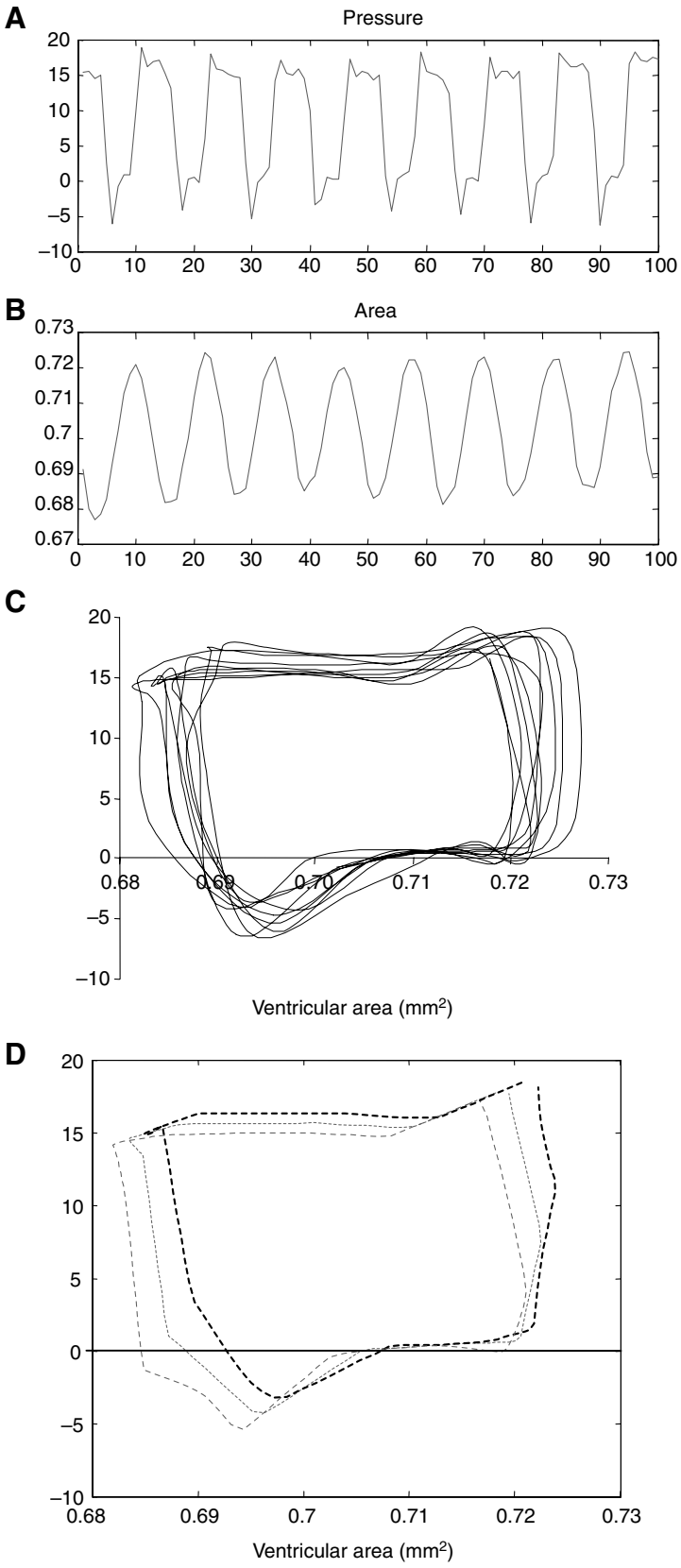

Fig. 5. (A) Pressure tracing. (B) Changes in area with each cardiac cycle calculated from ROI. (C) Eight PA loops generated by combining the values from A and B. (D) The result of MATLAB averaging of the eight loops and calculation of PA loop area.

Discussion

When comparing the PV loop of the grass shrimp ventricle (Fig. 6) to that of a multi-chambered vertebrate ventricle (Fig. 2), both PV loops contain the four primary phases of the cardiac cycle. The coordination of multiple outflow and inflow valves allows the single ventricle of the grass shrimp to generate discrete isovolumic contraction, ventricular emptying, isovolumic relaxation and ventricular-filling phases.

Although all four phases are present in the PA loop, the timing of the phases is considerably different than in the mammalian ventricle. In the mammalian four-chambered heart, approximately 33% of the cardiac cycle accounts for time in systole, with the remaining 67% in diastole. In this study we find that a much greater portion of the cardiac cycle is spent in systole (53%). In crayfish with heart rates in the 160–200 beats min^{-1} range, systole accounts for more than 60% of the cardiac cycle (Reiber, 1995). The negative filling pressure or diastolic sucking observed in this study may account for the reduction in diastolic filling time when compared with the filling times observed in the mammalian ventricles.

In a closed vertebrate circulatory system EDV is affected by venous filling pressure, distensability of the ventricular wall and time available for filling (Sagawa et al., 1988). When comparing ventricular EDV in an open circulatory system to that of a ventricular EDV in a closed circulatory system the most obvious difference is the effect of venous filling pressure. In the closed vertebrate system blood flows into the ventricle *via* a discrete pathway supplied by the vena cava, with the remainder of ventricular filling accomplished by contraction of the atria. In the open circulatory system of crustaceans there is no direct venous return path; nor as in multi-chambered hearts is there atrial contraction to enhance filling of the ventricle. Instead, filling occurs as a result of the pressure difference between the pericardial sinus and the expanding ventricle through the open ostia. During the cardiac cycle the ventral pericardial membrane is depressed during diastole and relaxes during systole, enhancing hemolymph flow from the branchio-cardial veins into the pericardial sinus (Belman, 1975; Reiber, 1994). The hemolymph in the pericardial sinus bathes the ventricle and then passively enters the relaxed ventricle through the open ostial valves.

At the onset of systole in the grass shrimp, the six ostial valves close, as evidenced by the rapid rise in pressure, with no change in volume. The ostial valves have an inward-pointing arrangement that prevents backflow during systole (Yazawa et al., 1999). In decapod crustaceans, there are seven arteries leaving the ventricle with outlets that are regulated by muscular bicuspid valves. The valves prevent passive reflux of hemolymph during diastole, but actively control outflow during systole *via* neural innervation (Alexandrowicz, 1932). Both excitatory and inhibitory neurons are present in the valves

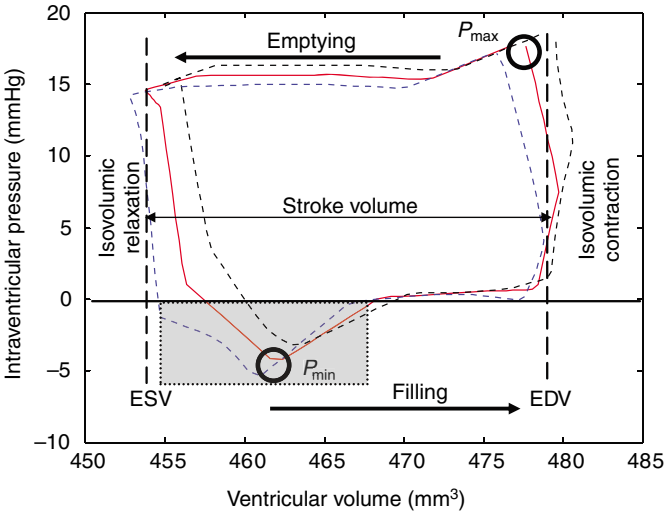

Fig. 6. Representative PV loop after area conversion and MATLAB analysis. EDV, end-diastolic volume; ESV, end-systolic volume; the grey shaded area is a period of ventricular 'sucking'.

(Kuromoto et al., 1992), with excitation causing valve muscle contraction that impedes flow and inhibition, causing relaxation that facilitates flow (Wilkens, 1997). Given that each of the valves is innervated, the ventricle must not only generate sufficient pressure to overcome resistance in the vasculature to open the valves (afterload), but the amount of resistance is also altered depending on the contractile state of the valves. The isovolumic contraction phase therefore requires the overcoming of peripheral resistance along with the nervous coordination of the timing and tension in the individual valves.

The emptying phase of the cycle is characterized by an initial drop in pressure, followed by a more stable pressure during the remainder of emptying in all PV loops that were analyzed. In macruran decapod crustaceans, between 50–60% of cardiac output is delivered to the large sternal artery, which travels ventrally and then branches in the anterior and posterior

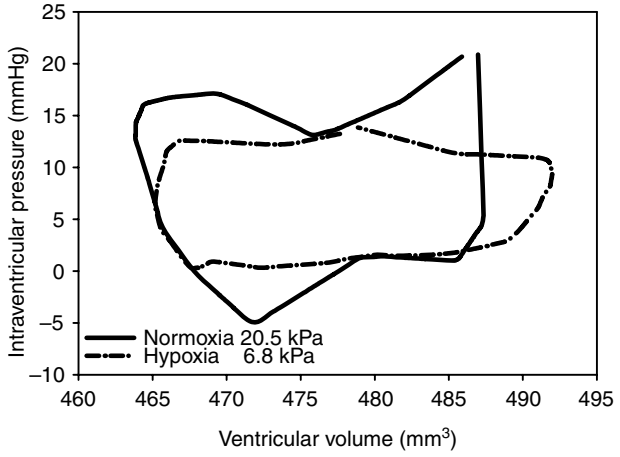

Fig. 7. An example of PV loops from one animal under normoxic conditions (20.5 kPa) and 30 min of hypoxia (6.2 kPa).

direction to supply the ventral nerve cord as well as other tissues (Fig. 1) (Reiber, 1994; Guadagnoli and Reiber, 2005). The sternal artery is the primary vessel responsible for the delivery of hemolymph to nervous tissue. Although we do not have specific data on the sequential opening of the arterial valves, the nature of the pressure tracings may be because of the independent neural innervation and timing of the valves. If the sternal artery were to open first, this could account for the drop in pressure associated with the first portion of the emptying phase. Thereafter, emptying of the ventricle occurs at a steady rate until the closing of the valves at the end of systole.

In a closed system, ventricular relaxation begins with an isovolumic phase with all valves closed and a rapid drop in pressure toward zero. This isovolumic relaxation phase in the ventricle of the grass shrimp continues until pressure falls below zero. As pressures drop, there is clear evidence of a 'diastolic sucking' phase as the ventricle begins to fill during negative pressure (Kraner, 1959) and completes its filling at low, but positive pressures. Negative pressures are not usually observed in crustaceans, with passive ventricular filling resulting from the pressure difference between the pericardial sinus and the ventricle (Belman, 1975; Reiber, 1994). Active diastolic sucking has been documented in mammalian ventricles and in chick hearts during development (Keller et al., 1990; Keller, 1994). In mature hearts negative pressures during ventricular filling are thought to result from restoring forces generated from the recoil of titin molecules within myocytes. A restoring force stores potential energy that can be converted to suction during the succeeding systole. In rat myocytes titin is responsible for ~90% of passive force during stretch and 60% of the restoring force (Helmes et al., 2003). Titin has been described in striated muscles of invertebrate species including crayfish (Fukuzawa et al., 2002), but its functional significance remains unclear.

The heart of decapod crustaceans also has an external mechanism for generating restoring forces. The decapod crustacean heart is held within the pericardial sinus *via* suspensory ligaments that stretch during systole and recoil during diastole. As suspensory ligament tension is increased, diastolic expansion enlarges because of greater elastic recoil (Volk, 1988). Although the ventricle begins to fill under negative pressure, the remainder of filling is accomplished *via* the pressure difference between the ventricle and the pericardial sinus. The ventricle of the grass shrimp may have both active (recoil) and passive (ΔP) properties available during the filling phase.

The area enclosed by the PV loop is an indicator of SW. Analysis of SW is useful in determining the efficiency of cardiac contraction and how this may change under various conditions. Based on the hypoxic PV loop, the pressure difference is decreased, stroke volume is increased and, overall, total SW is reduced (Fig. 7). During hypoxia, heart rate decreases, contributing to a decline in total CW. The fall in pressure may be the result of a decreased resistance in the branchial vasculature and a reduction in valve tension by the

nerves regulating the arterial valves. Stroke volume may be increased simply because of the increased amount of time available for filling or enhanced tension across the suspensory ligaments *via* the muscles attached to the epimeral wall. The future use of the PV loops in evaluating the cardiac response to stress, neurohormones and toxins will allow for a more detailed understanding of cardiac function than can be provided by independent measures of volume or pressure.

In multi-chambered hearts of closed systems, pressure and volume data have been used extensively to understand the mechanics and energetics of ventricular functions. Investigation of the decapod crustacean heart continues in an effort to obtain a clearer understanding of its filling and contractile properties. These investigations may be further enhanced using PV loops. Given the ongoing study of physiological stressors and interactions in this model, the use of PV loops provides a new tool for researchers to evaluate multiple levels of ventricular function in the open-circulatory system of decapod crustaceans.

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