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RESEARCH ARTICLE

Changes in cardiac performance during hypoxic exposure in the grass shrimp *Palaemonetes pugio*

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SUMMARY

In hearts of higher invertebrates as well as vertebrates, the work performed by the ventricle is a function of both rate and contractility. Decapod crustaceans experience a hypoxia-induced bradycardia that is thought to result in an overall reduction in cardiac work; however, this hypothesis has not yet been tested and is the primary purpose of this study. In the grass shrimp *Palaemonetes pugio*, cardiac pressure and area data were obtained simultaneously, and *in vivo*, under normoxic (20.2 kPa O_2) and hypoxic (6.8 or 2.2 kPa O_2) conditions and integrated to generate pressure–area (P–A) loops. The area enclosed by the P–A loop provides a measure of stroke work and, when multiplied by the heart rate, provides an estimate of both cardiac work and myocardial O_2 consumption. Changes in intra-cardiac pressure (d*p*/d*t*) are correlated to the isovolemic contraction phase and provide an indication of stroke work. At both levels of hypoxic exposure, intra-cardiac pressure, d*p*/d*t*, stroke work and cardiac work fell significantly. The significant decrease in intra-cardiac pressure provides the primary mechanism for the decrease in stroke work, and, when coupled with the hypoxia-induced bradycardia, it contributes to an overall fall in cardiac work. Compared with normoxic P–A loops, hypoxic P–A loops (at both levels of hypoxia) become curvilinear, indicating a fall in peripheral resistance (which might account for the reduction in intra-cardiac pressure), which would reduce both stroke work and cardiac work and ultimately would serve to reduce myocardial O_2 consumption. This is the most direct evidence to date indicating that the hypoxia-induced bradycardia observed in many decapod crustaceans reduces cardiac work and is therefore energetically favorable during acute exposure to conditions of low oxygen.

Key words: crustacean, cardiovascular, cardiac work, hypoxia, myocardia, pressure area loop.

INTRODUCTION

The hypoxia-induced bradycardia observed in many adult decapod crustaceans when exposed to conditions of low oxygen could be favorable energetically to the animal if overall cardiac work were reduced. Cardiac work is the energy expended (or work performed) by the ventricle and is a function of heart rate and cardiac contractility. Cardiac contractility is the amount of force generated by the myocardium to pump blood throughout the vascular system and is a direct function of stroke work. Hypoxic exposure in most adult decapod crustaceans results in a hypoxia-induced bradycardia that is hypothesized to reduce overall cardiac work (Guadagnoli et al., 2007). A reduction in cardiac work is an integrated function of a series of variables associated with the decapod cardiovascular system, including a reduced heart rate, stroke work and vascular resistance (arterial and venous or sinus resistances).

The cardiovascular system can be loosely defined as a four-part system with: (1) a pump (the ventricle) for generating force to move (2) blood or hemolymph through (3) a distribution pathway (arterial system) and (4) a return pathway (analogous to a venous system). As stated above, alterations in any of these components can alter the amount of work required of the pump (overall cardiac work). 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 the 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. Hemolymph enters the heart through three pairs of muscular ostia (Fig. 1B,C) and leaves the heart via six aortic valves that lead to five arterial systems (Fig. 1). Pre-branchial hemolymph from active tissue is collected in large, paired infrabranchial sinuses that deliver the hemolymph to the gills to become re-oxygenated. 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). Contraction of the ventricle then distributes the oxygenated hemolymph to the arterial systems that branch repeatedly to terminate and directly bathe the tissues (McLaughlin, 1983).

Cardio-respiratory responses to hypoxia vary among species depending on the mechanisms employed to maintain oxygen delivery to the tissues and include, but are not limited to, changes in heart rate (f_H), stroke volume (V_S), hemolymph flow,

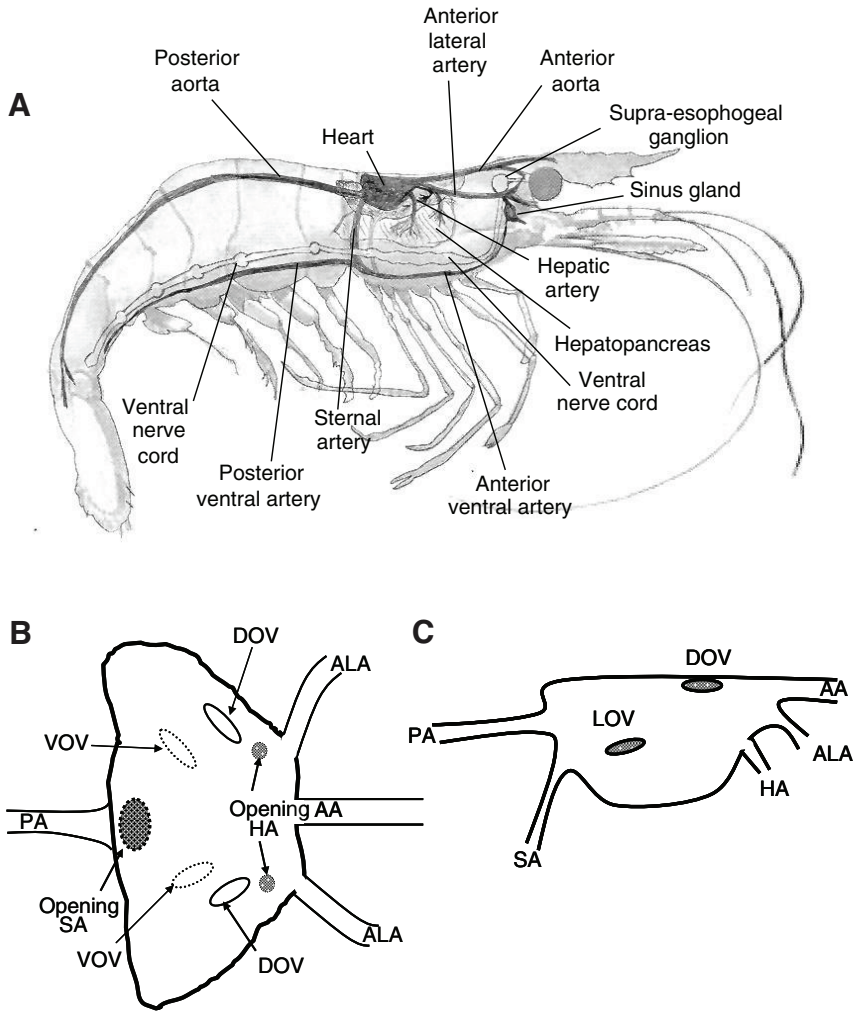

Fig. 1. (A) Overview of the circulatory anatomy of a shrimp. (B) Dorsal view of heart. (C) Lateral of view of heart. Abbreviations: AA, anterior aorta; ALA, anterior lateral artery; HA, hepatic artery; PA, posterior aorta; SA, sternal artery; VOV, ventral ostial valve; DOV, dorsal ostial valve; LOV,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

ventilatory rate and changes in the O_2 binding properties of respiratory pigments (McMahon, 2001; Reiber and McMahon, 1998; Wheatly and Taylor, 1981). In response to conditions of low oxygen, adult crayfish, crabs and lobsters typically exhibit a hypoxia-induced bradycardia with a concomitant increase in stroke volume (Wheatly and Taylor, 1981; Reiber, 1995; Reiber and McMahon, 1998; McMahon, 2001). The increase in stroke volume might result from the increased filling time that ultimately allows the animal to maintain cardiac output near normoxic values during exposure to hypoxia. Intracardiac, infra-branchial and pericardial sinus pressures have been shown to increase in crayfish upon exposure to hypoxia (Reiber et al., 1997). Although many studies have examined alterations in factors independent of each other upon exposure to hypoxia, the pressure, flow and volume data have been looked at independently and have not been integrated to determine whether these changes benefit the animal by reducing cardiac work. In the current study, in addition to changes in f_H, V_S, cardiac output ($\dot{V}_b$) and intra-cardiac pressure, we integrate the independent measures of ventricular area and intra-cardiac pressure to generate pressure–area (P–A) loops to determine changes in cardiac energetics during the response to hypoxia.

Changes in peripheral resistance within the arterial system also play a role in establishing the ventricular stroke work (or contractile force) required to move hemolymph through the system. Numerous studies have demonstrated that decapod crustaceans can redistribute hemolymph flow among their arterial systems (Guadagnoli and Reiber, 2005; McGaw et al., 1994; Reiber and McMahon, 1998). Redistribution of arterial flow is accomplished by muscular cardio-arterial valves located at the entrance to each arterial system (Maynard, 1960) and has been demonstrated in several species [in *Bathynomus*: by Kihara and Kuwasawa (Kihara and Kuwasawa, 1984); in *Cancer magister*: by McGaw and colleagues (McGaw et al., 1994); in *Panulirus japonicas*: by Kuramoto and Ebara (Kuramoto and Ebara, 1984); and in *Procambarus clarkii*: by Reiber (Reiber, 1994)]. Beyond the cardio-arterial valves, the vasculature has been shown to contain some contractile elements, yet the functional significance of this has yet to be determined (Shadwick et al., 1990; Wilkens, 1997; Wilkens and Taylor, 2003; Wilkens et al., 2008). One exception, the posterior aorta of the abdomen, contains muscle bands in its lateral walls and also has valves along its length, located at each of the branching segmental lateral vessels, that might allow for changes in resistance (Wilkens et al., 1997; Wilkens and Taylor, 2003). Muscular contractions of the abdomen (flexion and extension) might also provide a mechanism for altering vascular resistance (Reiber et al., 1997; Taylor, 1990). Thus, modulation of cardiac stroke work could result from any mechanisms affecting downstream resistance.

Here, we test the hypothesis that cardiac function will be altered to reduce cardiac work in animals exposed to hypoxia. We use independent measures of pressure and area to determine changes in intra-cardiac pressure, heart rate, stroke volume and cardiac output.

By integrating the pressure and area data, we generated P–A loops to evaluate changes in stroke work in the ventricle of the decapod crustacean *Palaemonetes pugio* (the grass shrimp) during hypoxic exposure.

MATERIALS AND METHODS

Animal preparation

Specimens of the grass shrimp *Palaemonetes pugio* Holthuis were purchased from GulfSpecimen Marine Laboratories and maintained in 20 l aquaria in aerated artificial seawater (30–32 ppt at 20°C, Instant Ocean, Blacksburg, VA, USA). Animals were maintained under laboratory conditions for two weeks before experimental use and were fed marine flakes (Tetra) three times a week. Experimental animals were placed into individual aquaria to separate them from the general population and fasted 2 days before use.

Grass shrimp (208.4±8.9 mg; N=22) 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 digital images of the heart could be captured through the transparent exoskeleton (Harper and Reiber, 1999; Harper and Reiber, 2001; Guadagnoli and Reiber, 2005; Guadagnoli et al., 2005a; Guadagnoli et al., 2005b).

Experimental design

Artificial seawater (Instant Ocean) circulated within a flow-through experimental chamber was maintained at 20°C, and water oxygen content was established and maintained using a gas mixing system (Cameron Instruments, Guelph, ON, Canada). All animals were initially placed in the experimental chamber in normoxic water (partial pressure of oxygen P_{O_2}=20.5 kPa) and allowed to settle for 1 h. A minimum of three recordings of pressure and volume were made for each animal during normoxic or 'control' conditions.

Hypoxic conditions (hypocapnic hypoxia) were achieved by controlled mixing of nitrogen and air using a gas mixing system (Cameron Instruments). The critical oxygen tension, or the P_{O_2} under which aerobic metabolism is no longer maintained, for *P. pugio* is reported to range from 4.7 (Harper and Reiber, 1999) to 5.3 kPa (Cochran and Burnett, 1996) – we therefore chose hypoxic conditions that would bracket this P_{crit} value. Water P_{O_2} in the flow-through chamber was lowered to either 6.8 kPa O_2 (N=12) or 2.2 kPa O_2 (N=10). Simultaneous measurements of intraventricular pressure and intraventricular area were made at 5, 10, 20 and 30 min after the onset of hypoxic exposure, which was then followed by a return to normoxia and a final measurement made at 35 min.

Intraventricular pressure

Intraventricular pressure was measured using a servo-null pressure system (model 900A, World Precision Instruments, Sarasota, FL, USA) and an analog-digital board (DAQPad 6020-50E, National Instruments, Austin, TX, USA) at a sampling rate of 600 Hz. Using low-compliance tubing and eliminating air bubbles (Fox and Wiederheilm, 1973), we obtained a frequency response of the hydraulic system of greater than 300 Hz. A glass micro-pipette with a 2.5 μm diameter tip was filled with 3 mol l^{-1} NaCl and positioned in the ventricle through the use of a micromanipulator (World Precision Instruments). The micro-pipette tip was inserted through the soft dorsal arthropodal membrane at the junction of the thorax and abdomen to minimize disturbance to the animal and then slowly advanced into the ventricle. The micro-pipette tip remained patent for over 45 min, after which clotting could become an issue. Insertion of the micro-pipette did not impede ventricular function, as indicated by consistence in both cardiac rhythm and cardiac function. The shrimp also showed no signs of struggling during the insertion of the micro-pipette tip. All animals survived the experiments, which indicated the minimal invasiveness of the technique. 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 as the difference between the measured pressure within the ventricle and the pressure recorded when the tip was placed in the experimental chamber at a level adjacent to the heart (Tobita and Keller, 2000).

Video image processing

Video images were acquired *in vivo* through the transparent exoskeleton of the shrimp at a rate of 60 Hz by using a stereomicroscope (Leica MZ12.5, McBain Instruments, Simi Valley, CA, USA) equipped with a video camera (World Precision Instruments), frame grabber board (LG-3, Scion, Frederick, MD, USA) and programmed image-acquisition software (Scion Image, Scion). Each video frame was analyzed to determine ventricular area by using custom-programmed image-analysis software (LabView, National Instruments, Austin, TX, USA) commonly used in the study of chick embryos (Tobita and Keller, 2000). Maximum and minimum ventricular borders were traced from recorded sequences to determine the ventricular longitudinal-sectional area (Fig. 2A,B). The number of pixels and individual pixel values in the area contained between the maximum and minimum borders were stored in memory as a 'region of interest' (ROI) (Fig. 2C). Assuming that movement of the ventricular border would be associated with changes in the pixel values within the image of the heart, changes in ventricular area from the minimum area during the cardiac cycle were identified automatically by detecting the pixels that changed in value within the defined ROI for sequential video fields. The total ventricular longitudinal sectional area in each video field was then calculated as the sum of the changes in area within the ROI and the minimum ventricular area. Ventricular area was used to construct P–A loops to remain consistent with established vertebrate protocols (Tobita and Keller, 2000), recognizing that dorsal–ventral movements of the heart (albeit very small as a proportion of the total volume change, as determined by the authors) might play a role in volume changes.

The pressure signal (600 Hz) (Fig. 2D) and video images (60 Hz) (Fig. 2E) were acquired simultaneously for ten periods of 4 s during each treatment sampling period using an output trigger to the AD board and the image-acquisition board. By using a custom computer program (K. Tobita using LabView, National Instruments), the pressure waveform was decimated from 600 Hz to 60 Hz and interpolated with the image data to yield a series of x–y coordinates required for the P–A loop (Fig. 2F).

Pressure and area analysis

A representative ventricular pressure tracing acquired at 600 Hz is shown in Fig. 2D. Peak and minimal ventricular pressure for each beat was used to calculate the mean maximal and minimal ventricular pressure, P_{max} and P_{min}. Heart rate was determined by measuring the average time between pressure peaks. The tracing in Fig. 2E is the result of digital image analysis using automated border detection in a defined ROI. The average of the area peaks (A_{max}) is equivalent to end-diastolic area (EDA), and the average of the area minima (A_{min}) is equivalent to end-systolic area (EDA). Plotting both the

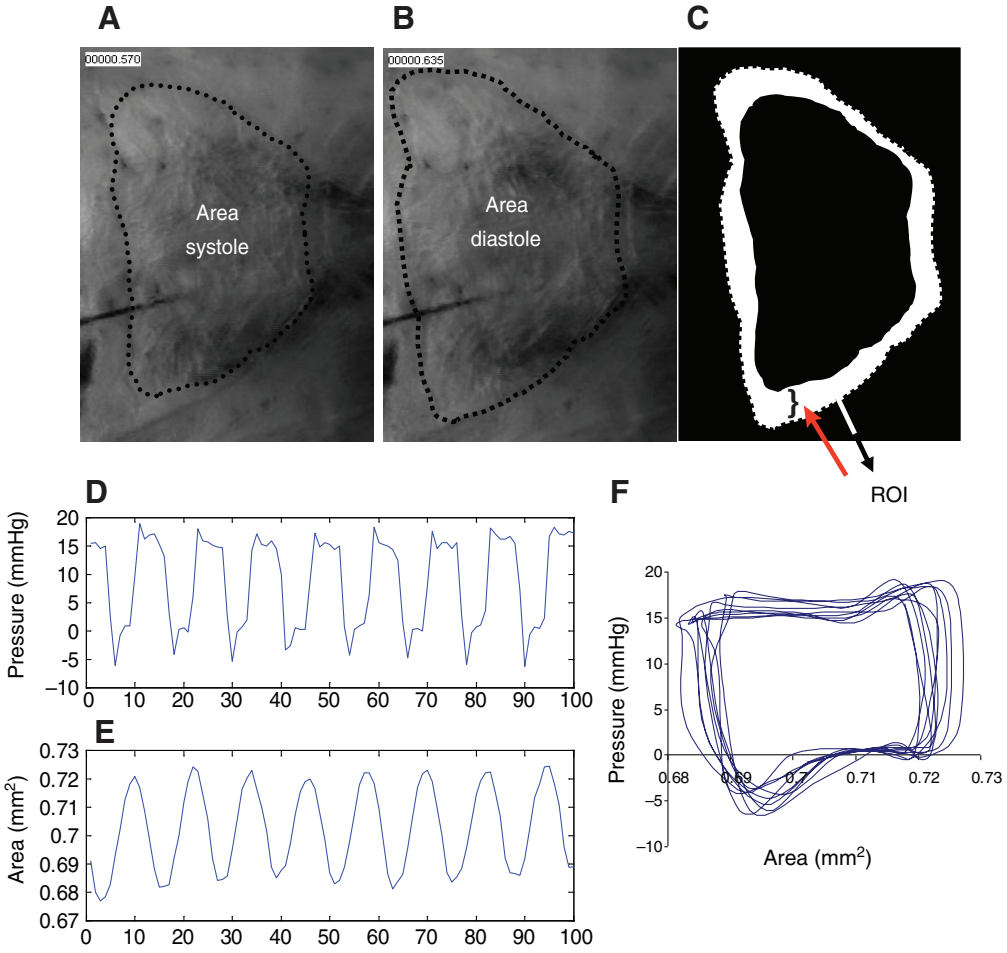

Fig. 2. (A) An outline of the heart in end systole as it defines the minimal area of the heart. (B) An outline of the heart in end diastole as it defines the maximal area. (C) The difference between the maximal (end-diastolic volume, EDV) and minimal (end-systolic volume, ESV) areas defines the region of interest (ROI) or stroke volume used in automated area analysis. (D) Intraventricular pressure, as it coordinate with E. (E) Changes in cardiac area (multiple cardiac cycles are shown) calculated from ROI. (F) Eight P–A loops generated by combining the values from D and E.

pressure and area data on a single *x*–*y* coordinate system yields eight P–A loops (Fig. 2F).

Statistical analysis

Repeated measures ANOVA was used to determine the time-course of changes in cardiac parameters from normoxic to hypoxic values (6.8 or 2.2 kPa O_2) after 5, 10, 20 and 30 min (Sigma Stat 9.0). If changes occurring in cardiac parameters were significantly different, multiple pair-wise comparisons were made using the Holm–Sidak method. Heart rate (f_H), maximum pressure (P_{max}), minimum pressure (P_{min}), change in pressure (ΔP), first derivative of maximal pressure (dp/dt), maximum area (A_{max}), minimum area (A_{min}) and change in area (ΔA) were determined by importing the pressure and video output from LabView (National Instruments) to MATLAB (The Mathworks, Natwick, MA, USA) by using a customized computer program (J. Vance). All values are presented as means ± s.e.m.

For the determination of stroke volume, A_{max} and A_{min} were converted to volume. The heart was modeled as a trapezoid [$d(0.5h(b+a))$, where d is depth, h is height, a is base length and b is top length] and ventricular volume was calculated as the product of depth (d) times ventricular area [$0.5h(b+a)$]. In previous studies, the depth (d) of the heart was determined to be $0.64h$ during systole and $0.67h$ during diastole (Harper and Reiber, 1999). Stroke volume was calculated as the difference between cardiac volumes (end systole – end diastole), and cardiac output was calculated as the product of heart rate and stroke volume.

After interpolation of the P–A data to generate multiple P–A loops in LabView, the data were analyzed using MATLAB to obtain an average P–A loop from five to 12 cardiac cycles as well as the area enclosed by the average P–A loop (Fig. 3). The area of the P–A loop is an estimate of stroke work (SW). Cardiac work (CW) is the product of SW per beat and heart rate.

RESULTS

Tables 1 and 2 provide a detailed summary of the time-course of changes in cardiac variables for 6.8 and 2.2 kPa O_2, respectively. In grass shrimp exposed to 6.8 kPa O_2, f_H falls significantly after 5 min of hypoxic exposure ($F_{4,59}$=8.52, P<0.001) and remains unchanged for the remainder of the 30 min exposure to hypoxic conditions (Fig. 4A). Changes in V_S are not statistically significant, yet the slight increase above normoxic values might be biologically relevant in helping maintain $\dot{V}_b$ (Fig. 4A) in the face of declining f_H. When grass shrimp are exposed to 2.2 kPa O_2 (Fig. 4B), f_H falls significantly after 5 min ($F_{4,49}$=25.7, P<0.001). Changes in V_S are not significant, and the significant drop in $\dot{V}_b$ ($F_{4,49}$=27.5, P<0.001) reflected the changes in f_H (Fig. 4b). After 20 min of exposure to hypoxia, there is a second drop in both f_H and $\dot{V}_b$. Recovery values (35 min) were obtained for several animals after a return to normoxia (6.8 kPa, N=4; 2.2 kPa, N=6). Although these values do not appear to be different from normoxic values at time 0 min, owing to the small number of samples, these data were not included in our statistical analysis (Fig. 4A,B).

Pressure changes are much more dramatic than changes in f_H, V_S or $\dot{V}_b$ at 6.8 kPa O_2. The fall in P_{max} ($F_{4,59}$=17.0, P<0.001) and increase in P_{min} ($F_{4,59}$=7.8, P<0.001) result in a decrease in intracardiac ΔP ($F_{4,59}$=23.2, P<0.001) after only 5 min of hypoxic exposure, with a second drop in ΔP after 10 min of hypoxic

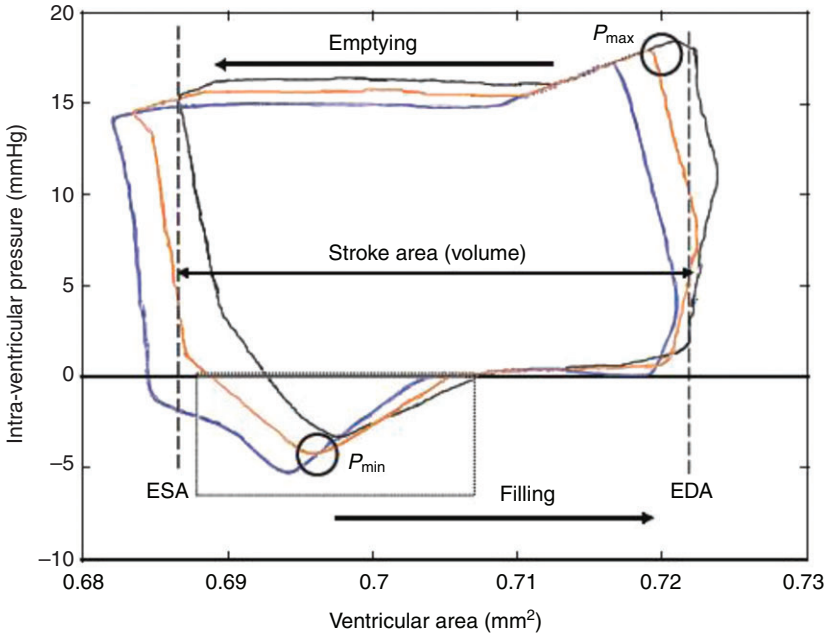

Fig. 3. Representative pressure–area (P–A) loops obtained from averaging eight P–A loops using MATLAB (each color represents an averaged P–A loop). ESA, end-systolic area; EDA, end-diastolic area. The box-enclosed area (lower left) outlines a period of ventricular 'sucking'. The circles delineate maximum pressure (P_{max}, upper right) and minimum pressure (P_{min}, lower left).

exposure (Fig. 5A). Interestingly, at 2.2 kPa O_2, the changes in ΔP ($F_{4,49}$=7.7, P<0.001) after 5 min result from changes in P_{max} only ($F_{4,49}$=19.2, P<0.001) (Fig. 5B). In both cases, values appear to return to normal after 5 min of normoxia (Fig. 5A,B).

Maximal ventricular dp/dt drops significantly from normoxic values after 5 min of hypoxic exposure (Fig. 6) at both 6.8 kPa O_2 ($F_{4,59}$=18.1, P<0.001) and 2.2 kPa O_2 ($F_{4,49}$=18.6, P<0.001).

The area within the P–A loop is a measure of stroke work (SW). Representative examples of P–A loops for both 6.8 and 2.2 kPa O_2 are given in Fig. 7A and Fig. 8A. At 6.8 kPa O_2, mean P–A loop area does not drop significantly; however, cardiac work (CW), the product of f_H and P–A area (SW), falls significantly ($F_{4,59}$=5.8, P<0.001) after 20 and 30 min of exposure to 6.8 kPa O_2 (Fig. 7B), corresponding with the decrease in f_H. At 2.2 kPa O_2 (Fig. 8B), CW

Table 1. Changes in cardiovascular parameters upon exposure to 6.8 kPa

Parameter	Normoxia (20.2 kPa)	5 min 6.8 kPa	10 min 6.8 kPa	20 min 6.8 kPa	30 min 6.8 kPa
Heart rate (beats min^{-1})	285±13	263±13*	260±12*	260±12*	253.0±11*
End-diastolic volume (μl)	572.8±14.5	595.2±17.8	573.1±18.8	566.9±19.4	563.3±22.3
End-systolic volume (μl)	470.6±12.8	487.7±15.2	466.4±18.0	463.7±18.9	459.4±21.3
Stroke volume (μl beat^{-1})	102.2±3.2	107.4±3.7	106.7±2.3	103.2±2	103.9±1.9
Cardiac output (ml min^{-1})	29.04±1.5	28.0±1.3	27.7±1.5	26.9±1.4	26.08±4.9
P_{max} (mmHg) (peak systolic pressure)	20.9±1.3	18.1±1.4*	16.3±1.3*	16.2±1.1*	16.1±1.1*
P_{min} (mmHg) (minimal diastolic pressure)	–7.5±0.7	–6.4±0.3*	–6.2±0.3*	–5.9±0.5*	–6.0±0.7*
ΔP (mmHg) (change in pressure)	29.4±1.8	24.3±1.6*	22.6±1.4*	22.1±1.3*	22.2±1.1*
dp/dt_{max}	3.8±0.6	2.7±0.4*	2.2±0.4*	2.3±0.4*	2.3±0.5*
Stroke work P–A loop area (mm^2 mmHg)	0.75±0.14	0.76±0.2	0.68±0.1	0.61±0.1	0.63±0.37
Minute cardiac work (mm^2 mmHg min^{-1})	213.0±29.4	197.1±33.9	171.8±29.2	153.1±28.1*	153.3±21.4*

The data are expressed as means ± standard error of the mean. *Statistical significance was assigned based on P<0.05 (N=12; mean animal mass 204.2±9.2 mg).

Table 2. Changes in cardiovascular parameters upon exposure to 2.2 kPa

Parameter	Normoxia (20.2 kPa)	5 min 2.2 kPa	10 min 2.2 kPa	20 min 2.2 kPa	30 min 2.2 kPa
Heart rate (beats min^{-1})	277±3	244±9*	224±8*	203±7*	203.4±7.4*
End-diastolic volume (μl)	644.8±38.3	657.8±31.5	667.6±33.0	662.3±33.4	646.7±31.3
End-systolic volume (μl)	510.3±30.8	520.6±26.7	530.4±28.4	524.8±27.0	522.2±27.9
Stroke volume (μl beat^{-1})	134.5±8.2	139.2±7.8	137.2±6.4	137.4±8.4	124.6±5.7
Cardiac output (ml min^{-1})	37.2±1.52.3	34.6±2.3*	31.2±41.8*	28.4±2.2*	25.4±1.7
P_{max} (mmHg) (peak systolic pressure)	24.0±2.5	18.7±2.0*	18.4±2.6*	17.7±2.7*	17.0±2.5*
P_{min} (mm Hg) (minimal diastolic pressure)	–6.2±0.6	–5.3±1.1	–6.0±1.2	–5.9±1.3	–6.30±1.4
ΔP (mmHg) (change in pressure)	31.1±3.1	23.0±1.8*	24.4±2.2*	23.5±2.3*	23.5±1.8*
dp/dt_{max}	3.8±0.5	2.0±1.6*	1.8±0.2*	1.5±0.2*	1.6±0.3*
Stroke work P–A loop area (mm^2 mmHg)	1.21±0.15	1.01±0.13	0.87±0.09*	0.80±0.09*	0.68±0.07*
Minute cardiac work (mm^2 mmHg min^{-1})	332.6±37.8	241.1±27*	193.9±21*	165.4±2.2*	140.8±17.9*

The data are expressed as means ± standard error of the mean. *Statistical significance was assigned based on P<0.05 (N=10; mean animal mass 213.4±16.6 mg).

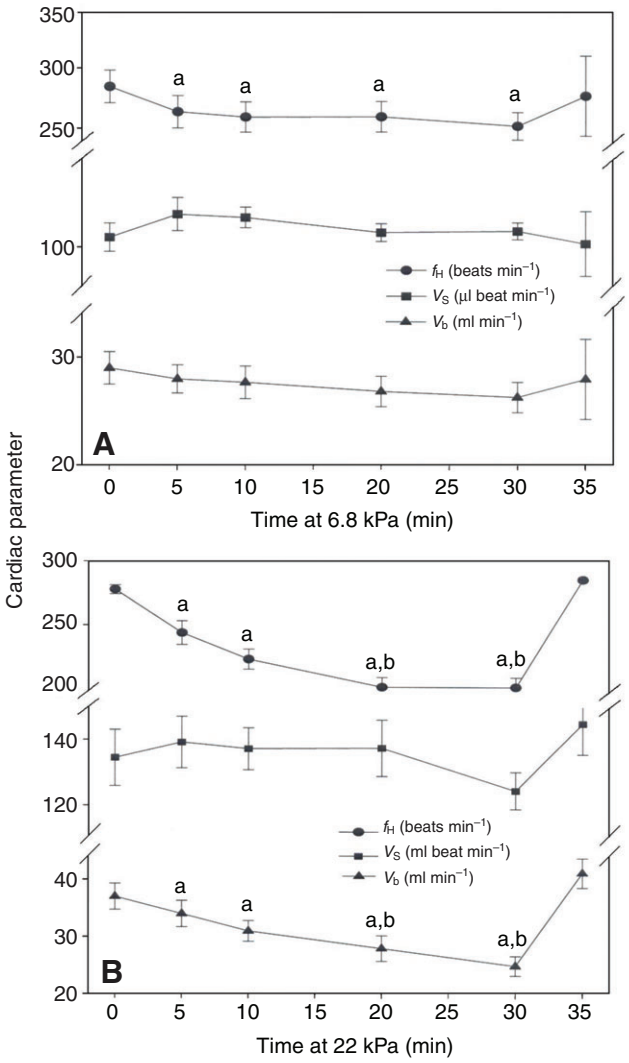

Fig. 4. Cardiac parameters obtained from shrimp exposed to hypoxic conditions of (A) 6.8 kPa (N=12) and (B) 2.2 kPa (N=10). Measurements were made at 5, 10, 20 and 30 min post exposure to hypoxic conditions, which was followed by exposure to normoxic water for 5 min, and a final measurement taken at 35 min. The letter 'a' denotes a standard deviation (s.d.) from time 0 at 20.5 kPa; 'b' denotes a s.d. from 10 min (P<0.001).

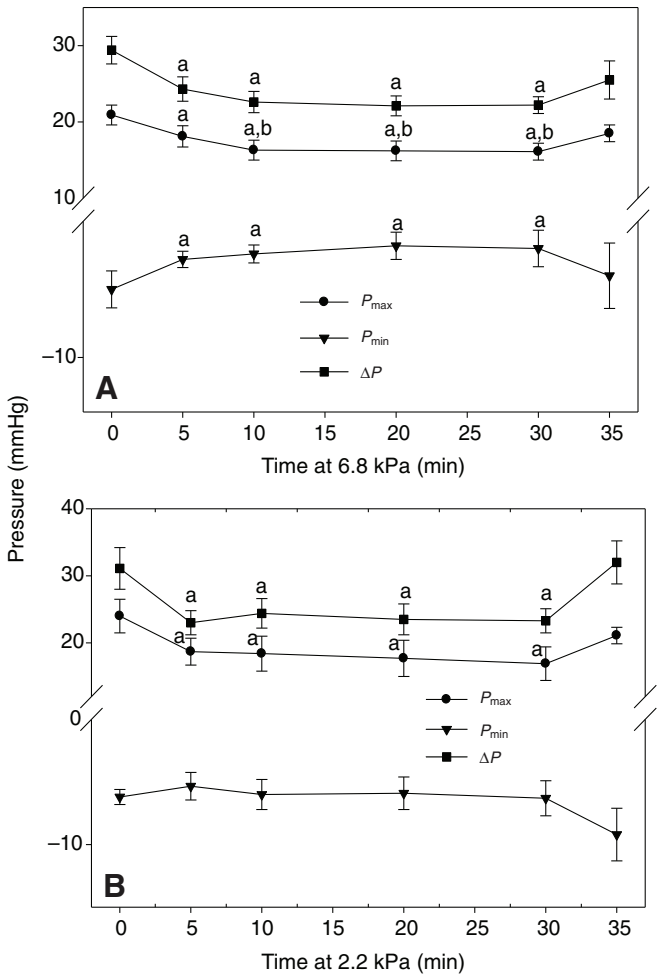

Fig. 5. Pressures obtained from shrimp exposed to hypoxic conditions of (A) 6.8 kPa (N=12) and (B) 2.2 kPa (N=10). The letter 'a' denotes a standard deviation (s.d.) from time 0 at 20.5 kPa; 'b' denotes a s.d. from 10 min (P<0.001).

drops significantly ($F_{4,49}$=33.8, P<0.001) after 5 min of hypoxic exposure owing to the previously mentioned drop in f_H, coupled with the significant fall in the P–A loop area ($F_{4,49}$=13.6, P<0.001).

DISCUSSION

Within the single ventricle of the grass shrimp *P. pugio*, the coordinated opening and closing of multiple inflow and outflow valves allows the ventricle to function in a manner similar to the ventricles of vertebrate multi-chambered hearts. The P–A loop of the grass shrimp (Fig. 3) has the same four primary phases as P–A loops generated in the ventricle of a closed system: (1) isovolemic contraction, (2) ventricular emptying, (3) isovolemic relaxation and (4) ventricular filling. The loop can therefore be used to determine changes in cardiac energetics during hypoxic stress. The hypoxia-induced bradycardia and the fall in intra-cardiac pressure contribute to a reduction in overall cardiac work during hypoxic exposure.

Changes in f_H, V_S, $\dot{V}_b$ and pressure

As with many adult decapod crustaceans, grass shrimp exhibit a hypoxia-induced bradycardia. In these animals, we did not observe an increase in V_S as expected, despite the maintenance of $\dot{V}_b$ at 6.8 kPa O_2 (Wheatly and Taylor, 1981; McMahon and Wilkens, 1975; Reiber and McMahon, 1998). In previous studies, when grass shrimp were exposed to 2 h of hypoxia at a level of 6.8 kPa O_2 (Guadagnoli and Reiber, 2005), and in this study, $\dot{V}_b$ was maintained near normoxic values with increases (although not statistically significant) in stroke volume. At 2.2 kPa O_2, a value well below the P_{crit} value of the animal (Harper and Reiber, 1999; Cochran and Burnett, 1996), grass shrimp were unable to maintain cardiac output, with the fall in f_H not supported by sufficient increases in V_S. In a small number of samples (N=6), recovery data were available. Although cardiac output drops quickly at 2.2 kPa O_2, cardiac parameters appear to return to their original normoxic values after a 5 min normoxic recovery period. Although 2.2 kPa O_2 is below the P_{crit} value of the grass shrimp, a period of 30 min of hypoxic stress is not sufficient to hinder the ability of the grass shrimp to recover.

Intra-cardiac pressure has been measured previously in decapod crustaceans weighing from 12 g (*Gnathophausia ingens*) to over

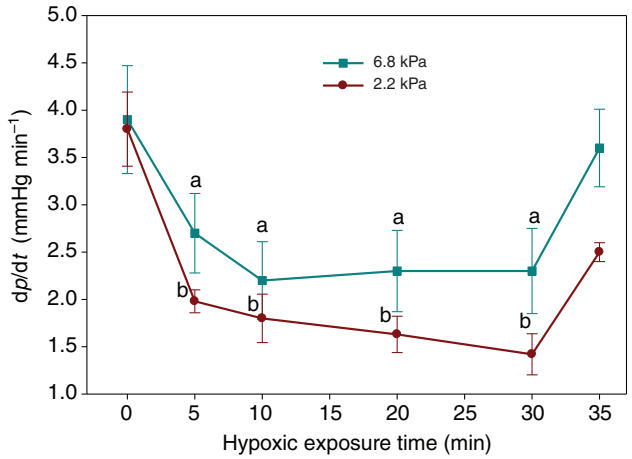

Fig. 6. Changes in ventricular dp/dt at 6.8 and 2.2 kPa O_2. The letter 'a' denotes values at 6.8 kPa that are a standard deviation (s.d.) from normoxic values. The letter 'b' denotes values at 2.2 kPa that are a s.d. from normoxic values. Measurements were made at 5, 10, 20 and 30 min post exposure to hypoxic conditions, which was followed by exposure to normoxic water for 5 min, and a final measurement taken at 35 min.

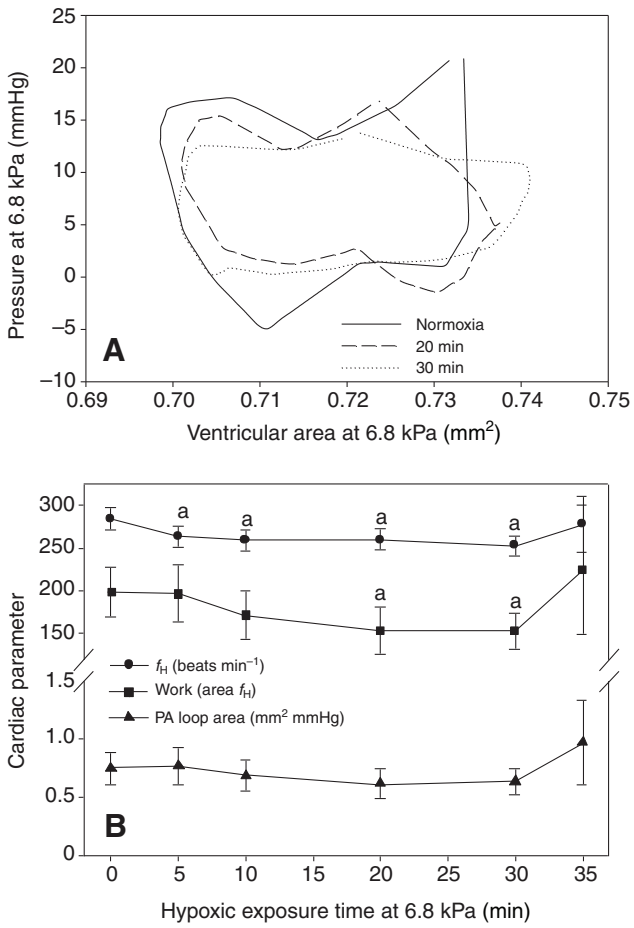

Fig. 7. (A) Representative pressure–area (P–A) loops from a single animal under normoxic conditions (20.5 kPa) and after 20 and 30 min of hypoxia at 6.8 kPa. The P–A loop areas are 0.5682, 0.5092 and 0.5168 for normoxia and 20 and 30 min at 6.8 kPa, respectively. (B) Relationship between f_H, P–A loop area and minute cardiac work (CW). Measurements were made at 5, 10, 20 and 30 min post exposure to hypoxia, which was followed by exposure to normoxic water for 5 min, and a final measurement taken at 35 min. The letter 'a' denotes a standard deviation (s.d.) from time 0 (control 20.5 kPa) at 6.8 kPa (P<0.001); the letter 'b' denotes a s.d. from time 0 (control 20.5 kPa) at 2.2 kPa (P<0.001).

500 g (*Cancer antennarius*, *Homarus americanus*) (Belman, 1975; Reiber and McMahon, 1998), with mean pressures ranging from 6.2 to 36.3 mmHg during systole, 1.6 to 22.1 mmHg during diastole and f_H from 90–180 beats min^{-1} (Belman, 1975). In this study, we measured intra-cardiac pressure *in vivo* in a milligram-sized decapod crustacean. Maximal diastolic intra-cardiac pressure and ΔP dropped rapidly upon exposure to both levels of hypoxia. Intra-cardiac, infra-branchial and pericardial sinus pressures have been shown to increase upon exposure to hypoxia in crayfish (Reiber and McMahon, 1998). However, a fall in intra-cardiac pressure alone during exposure to hypoxia would increase the passive ΔP for filling of the ventricle. Although we did not measure pericardial sinus pressures, Wilkens and McMahon (Wilkens and McMahon, 1992) have found that a fall in pericardial sinus pressure does not change end-diastolic volume. Overall, any increase in the passive ΔP might serve to reduce SW.

dp/dt_{max} is a descriptor of isovolemic pressure, and a fall dp/dt_{max} is associated with a fall in myocardial O_2 consumption (Saguwa et al., 1988; Senzaki et al., 2001). A fall in dp/dt_{max} was observed during both levels of hypoxic exposure, with a more dramatic fall during severe hypoxia. As the ventricle and the cardiac ganglion are highly aerobic organs, any conservation of myocardial O_2 consumption would be beneficial when an animal is exposed to a low-oxygen environment. The reduction in myocardial O_2 consumption is supported by the decrease in SW, as determined from P–A loops. Pressure–area loops from animals exposed to both levels of hypoxia show that the decrease in SW results primarily from the dramatic fall in ΔP.

The cardiac cycle and pressure–area loops

During the cardiac cycle, the ventral pericardial membrane is depressed during diastole, and, during systole, it returns to its previous placement, which creates pressure changes that enhance hemolymph flow from the branchio-cardial veins into the pericardial sinus (Belman, 1975; Reiber, 1994). Hemolymph in the pericardial sinus then enters the relaxed ventricle through the open ostial valves. At the onset of ventricular systole, the ostial valves close. The ostial valves have an inward-pointing arrangement that prevents backflow during systole (Yazawa et al., 1999).

At the onset of the isovolemic contraction phase, the ostial valves and arterial valves are closed. In decapod crustaceans, flow to the seven arteries leaving the ventricle is regulated by six muscular bicuspid valves. The valves prevent passive reflux of hemolymph during diastole but actively control outflow during systole by means of neural innervation (Alexandrowicz, 1932; Kuromoto and Ebara, 1984). Both excitatory and inhibitory neurons are present in the valves (Kuromoto et al., 1992). Neural excitation causes valve muscle contraction to impede flow, and neural inhibition causes relaxation to facilitate flow (Wilkens, 1997). The ventricle must not only generate sufficient pressure to overcome resistance within the vasculature (afterload), but the force required can also be altered depending on the contractile state of the valves. The isovolemic contraction phase therefore requires the overcoming of peripheral resistance along with the nervous coordination of the timing and tension in the individual valves.

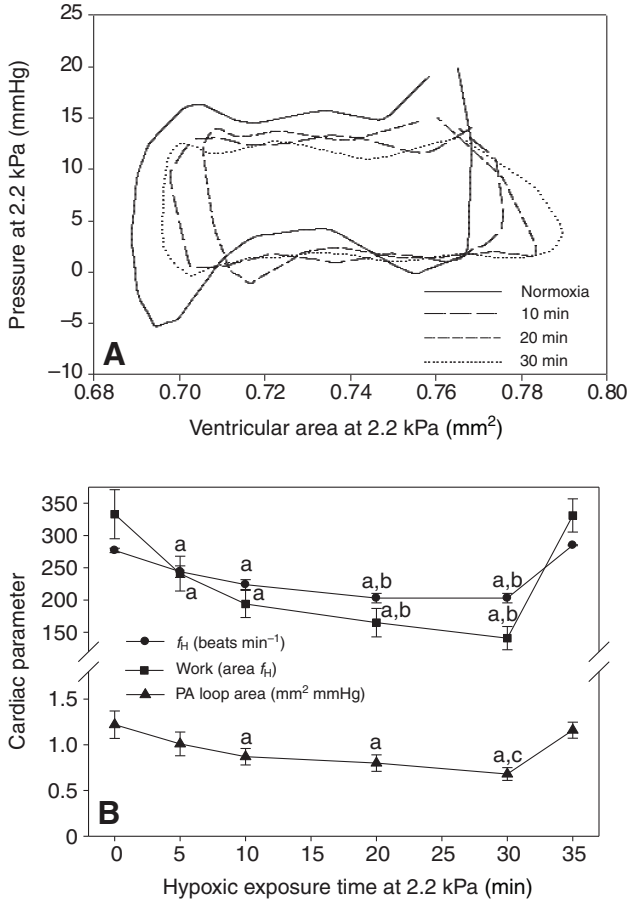

Fig. 8. (A) Representative pressure–area (P–A) loops from a single animal under normoxic conditions (20.5 kPa) and after 10, 20 and 30 min of hypoxia at 2.2 kPa. The P–A loop areas are 1.175 for normoxia and 0.867 at 10 min, 0.854 at 20 min and 0.848 at 30 min exposure to hypoxia (2.2 kPa). (B) Relationship between f_H, P–A loop area and minute cardiac work (CW). Measurements were made at 5, 10, 20 and 30 min post exposure to hypoxia, which was followed by exposure to normoxic water for 5 min, and a final measurement taken at 35 min. The letter 'a' denotes a standard deviation (s.d.) from time 0 at 20.5 kPa; the letter 'b' denotes a s.d. from 5 min at 2.2 kPa; and the letter 'c' denotes a s.d. from 10 min at 2.2 kPa (P<0.001).

During the relaxation phase, pressure approaches zero and becomes sub-ambient (negative relative to the pericardial sinus), and there is a period of 'diastolic sucking' as the ventricle begins to fill during sub-ambient pressure (Kraner, 1959) and completes its filling at low, but positive, pressures. Sub-ambient pressures have not yet been reported 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., 1991; Keller, 1994). The heart of decapod crustaceans has an external mechanism to generate restoring forces. The heart is held within the pericardial sinus by suspensory ligaments that stretch during systole and recoil during diastole. Although the ventricle begins to fill under sub-ambient pressure, the remainder of filling is accomplished by means of the pressure difference between the ventricle and the pericardial sinus. The ventricle of the grass shrimp would appear to have available both active (recoil) and passive (ΔP) properties during the filling phase, which might account for the observed negative pressures and resulting diastolic sucking.

The area enclosed by the P–A loop is an indicator of stroke work (SW). Analysis of SW is useful in determining the efficiency of the cardiac contraction and how this might change under various conditions (Sagawa et al., 1988; Guadagnoli et al., 2007). The significant fall in SW at 2.2 kPa O_2 appears to be largely due to the large drop in ventricular pressure as there are no significant changes in V_S. The declining trend in SW was not significant at 6.8 kPa O_2; however, when heart rate is considered, CW (min^{-1}) drops significantly. These data support a decrease in myocardial O_2 consumption during hypoxic exposure.

Two plausible explanations for the possible reduction in myocardial O_2 consumption are discussed: a fall in peripheral resistance and/or a change in suspensory ligament tension. The contraction and relaxation of the abdominal musculature and/or the extension or retraction of the tail region (Taylor, 1990; Reiber et al., 1997) can modulate peripheral vascular resistance. An extension of the tail region and increased pleopod fanning have been observed in grass shrimp exposed to severe hypoxia (Guadagnoli and Reiber, 2005). The ejection curve of grass shrimp P–A loops generated during exposure to hypoxia becomes progressively curvilinear, which is consistent with a reduction in vascular resistance (Keller et al., 1991). This would decrease the necessary amount of force generated by the ventricle to overcome resistance, thereby reducing myocardial O_2 consumption.

Elastic recoil of the suspensory ligaments that hold the heart within the pericardial chamber of decapods might also contribute to a conservation of myocardial O_2 consumption. End-diastolic volume is determined by the amount of elastic recoil in the suspensory ligaments (Rose et al., 2001). In addition to connective tissue, these ligaments contain innervated muscle fibers that attach to the epimeral wall (Volk, 1988). A manual increase in suspensory ligament tension results in a decrease in heart rate and an increase in diastolic expansion owing to greater elastic recoil. The stretch receptors that cause recruitment of the cardio-inhibitory nerve are located on the surface of the epimeral wall at the origin of the suspensory ligaments (Volk, 1988). An increase in tension in the muscles within the suspensory ligaments would increase ligmental tension. During ventricular contraction, the ligaments would be stretched more, storing more energy within them that can be recovered during elastic recoil to enhance diastolic filling (Volk, 1988). The oxygen demand of these small muscles would be substantially less than the oxygen consumption of the ventricle and might provide an additional mechanism for a reduction in myocardial O_2 consumption.

Although changes in V_S were not significant, the slight increases might be biologically relevant in allowing the animal to maintain $\dot{V}_b$. The accompanying decrease in f_H allows for increased filling time and reduces CW. The combination of these factors serves potentially to reduce myocardial O_2 utilization during exposure to 6.8 kPa O_2 while still maintaining $\dot{V}_b$. The drop in myocardial O_2 consumption at 2.2 kPa O_2 might result from the same factors noted previously. Hypoxic stress at 2.2 kPa O_2 is well below the P_{crit} value for grass shrimp, and so the animal might be forced to reduce overall metabolism, as evidenced by the significant drop in $\dot{V}_b$.

In multi-chambered hearts of animals with closed systems, pressure and area data have been used extensively to understand the mechanics and energetics of ventricular function. Continued use of P–A loops in this model will provide a greater understanding of ventricular function and its relationship to volume loading, osmotic balance, neurohormones and the work

performed by the ventricle under different physiological and biological stresses. Given the complex cardiac dynamics of the single ventricle of decapod crustaceans, the use of P–A loops will provide a gateway to a more complete understanding of ventricular energetics in this model.

LIST OF ABBREVIATIONS

CW	cardiac work
EDV	end-diastolic volume
ESV	end-systolic volume
f_H	heart rate
P–A	pressure–area
SW	stroke work
V_S	stroke volume
$\dot{V}_b$	cardiac output

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