Short-term compensatory and long-term plastic cardiorespiratory responses to hypoxic exposure

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SHORT-TERM COMPENSATORY AND LONG-TERM
PLASTIC CARDIORESPIRATORY RESPONSES
TO HYPOXIC EXPOSURE

By

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A dissertation submitted in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy in Biological Sciences
Department of Biological Sciences
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Graduate College
University of Nevada, Las Vegas
May 2006
Dissertation Approval
The Graduate College
University of Nevada, Las Vegas

April 12, 2006

The Dissertation prepared by

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Entitled

Short-term compensatory and long-term plastic cardiorespiratory responses to hypoxic exposure

is approved in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Biological Sciences

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ABSTRACT

Short-term Compensatory and Long-term Plastic Cardiorespiratory Responses to Hypoxic Exposure

by

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Dr. Carl L. Reiber, Examination Committee Chair
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Aquatic organisms may encounter multiple perturbations in their environment including but certainly not limited to fluctuations in temperature, salinity, conductivity, pH, carbon dioxide and oxygen content. A large body of work has elucidated the complexity of the cardiovascular system of crustaceans but the intricacies associated with alterations in cardiovascular function with changing environments are elusive. The current body of work focuses on the cardiovascular adjustments that allow crustaceans to survive decreases in oxygen tension.

The complexity of control of the open circulatory system of crustaceans has long been underestimated and the marine crustacean, the grass shrimp, *P. pugio*, which often encounters fluctuations in water oxygen content served as a subject of the following studies. Crustacea posses multiple outlets from their single chambered hearts, yet the vessels supplied by the heart are not muscular and are not able to alter resistance as in mammalian systems. Instead they have
muscular cardio-arterial valves that are innervated and the aperture of the valves is regulated by the central nervous system and several hormones.

The first goal of this body of work was to examine alterations in cardiovascular parameters which included heart rate, stroke volume, cardiac output and hemolymph flow, in different reproductive states, in response to declining oxygen tension. The data clearly demonstrate that as oxygen tension falls, even as cardiac output is maintained, there are significant alterations in flow. In particular, despite requiring flow to the ovary in reproductively active females, the metabolically demanding ventral nerve cord receives the greatest percentage of cardiac output and this percentage increases during hypoxic exposure while other less metabolically demanding areas of the animal receive reduced flow.

To appreciate the cardiac mechanics and energetics involved in the hypoxic response, these are the first studies to utilize pressure-area loops in the ventricle of an open circulatory system. Once we established that P-A loops were adequate to evaluate cardiac energetics, we used P-A loops to examine changes in ventricular function in response to hypoxia. Stroke work of the ventricle decreases significantly upon hypoxic exposure primarily as a result of reduced intra-cardiac pressure.

The second goal of the work was to describe alterations in oxygen binding pigments as animals are reared in or exposed to hypoxia. Both rearing in hypoxia and prolonged exposure to hypoxia as an adult led to significant increases in Hb concentration and altered Hb subunit expression. These changes are not
reversed in hypoxic reared adult animals indicating that the response is plastic during development and inducible in adulthood.

Collectively these data indicate that substantial but sustainable shifts in cardiovascular and cardiorespiratory parameters occur when these animals are exposed to hypoxia. Future research will continue to elucidate and clarify the suite of complex mechanisms involved in these responses.
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AKNOWLEDGEMENTS

Where does one begin to thank the many people whose influence has culminated in a life challenging and life changing experience like the completion of a scientific dissertation. I don’t believe anyone can truly respect and understand their predecessors until, finally one day you too stand as their peer. I must begin by thanking my advisors. After completing my master’s degree in exercise science, my major professor, Dr. Carl Reiber, assured me that moving from the Kinesiology Department to the Biology Department would not be a problem, “after all” he said, “physiology is the same in all organisms.” Being a naïve student I believed him...I definitely had a bit of learning curve coming into an invertebrate world. One thing I’ll give Carl is that he is PATEINT! Not only did I have lot to learn, I chose this time in my life get married and give birth to our two children (more on them later). Carl has been both a mentor and a friend, depending on what stage in the process we have been in together. I fought my battles, some won, and some lost, and in the end always learned something... Most of the time Carl just let me go and I am thankful to have learned that this path was indeed for me to find.

I must also thank the rest of committee, Steve Roberts, Iain McGaw and Bryan Spangelo. They each contributed a unique aspect to my progress. Steve was my brutally honest reality check. Steve could be my harshest critic, but it was always constructive, not destructive. He was also full of praise, and when
that came my way I always took pride in knowing he was genuinely proud or excited about what he was sharing with me. With Steve, I always knew what I was getting and I am so thankful for that kind of honesty...its hard to find. Iain was always supportive of my progress. He was always very encouraging, open and helpful. He would give praise even when I probably didn't deserve it. Finally, Byran Spangelo, the king of biochemistry. I never dreamed that being a physiologist would require such an in-depth knowledge of biochemistry, but I took more Biochemistry classes that I can count from Dr. Spangelo and never fell asleep. Bryan has been a supportive and rational outside committee member, especially when I felt the end would never happen. I thank all of you for your mentoring, your collegiality and your friendships.

I have shared the lab with too many graduate and undergraduate students to name them all, but I would like to thank Dr. Alyssa Braun, Dr. Stacy Harper and Dr. Karren Knerr in particular for their friendship, support and collegiality. Dr. Frank van Breukelen for his insights and encouragement and Mrs. Kate Shen for her valuable help in the molecular biology lab. Finally, I must thank my colleagues at Touro University in particular Dr. Mitchell Forman and Dr. Micheal Harter. They have stood by me for the last 20 months anxiously awaiting my completion, yet being patient none the less.

On a personal note I must thank my family. My father passed away during my first year as a PhD student, but I must thank both of my parents for their ongoing support. Finally I must thank my husband, Dr. Mark Guadagnoli. He has been a mentor, a sounding board and my constant support throughout this
process. He and our two wonderful children have been loving and patient in my completion of this sometimes arduous task.
CHAPTER 1

THE CARDIOVASCULAR RESPONSES TO HYPOXIA IN CRUSTACEANS

Introduction

Many aquatic organisms routinely encounter environments that fluctuate in their oxygen (O₂) availability, from normal levels of oxygen to hypoxic or nearly anoxic levels. This can be due to a number of factors starting with the physical characteristics of water. Water has a low oxygen capacitance, low oxygen diffusion and is more viscous than air (Dejours, 1989) all of which make oxygen more difficult to acquire for aquatic organisms. These characteristics are altered further when examining the differences between distilled water and waters of varying salinities. Increasing salinity and ion concentration increases the viscosity of water and further depresses O₂ carrying capacity providing an additional O₂ uptake challenge for organisms living in salt water versus fresh water.

Additionally, biotic factors such as the contribution of O₂ produced from photosynthetic organisms during the day may cause a sharp decrease in water oxygen partial pressure (PO₂) at night when photosynthesis no longer occurs. Oxygen partial pressure can be altered often times within a day, or may be changed more permanently due to an algal bloom, for example (Dejours, 1989). As a result, animals within these aquatic environments must have in place both
short-term and long-term compensatory mechanisms for dealing with alterations in \( \text{PO}_2 \).

In the past, animals have been divided into two categories in an effort to describe their oxygen tolerance: oxygen regulators and oxygen conformers. Oxygen regulators maintain oxygen consumption independent of \( \text{PO}_2 \) while oxygen conformers alter oxygen consumption in proportion to water \( \text{PO}_2 \) (Herried, 1980). However, it is important to realize that these two classifications sit at the extreme ends of possible physiological responses that animals may have in their arsenal of responses to hypoxia. Many animals are able to regulate oxygen consumption independent of water \( \text{PO}_2 \) down to some critical level at which point they are no longer able to match oxygen supply to demand and become oxygen conformers. This point, known as \( P_{\text{crit}} \), is a valuable tool used conceptually to evaluate and compare an animal's hypoxic response. Values for \( P_{\text{crit}} \) are variable within a species and differences can occur due to physical (temperature, salinity) and physiological (reproductive, metabolic, cardio-respiratory states) differences (Herried, 1980).

Crustaceans typically attempt to maintain oxygen consumption during hypoxia via physiological adjustments that lead to increased oxygen conductance such as ventilatory and cardiovascular adjustments as well as alterations in oxygen binding pigments. The main focus of this dissertation is to further our understanding of the compensatory mechanisms available to organisms by examining the cardiovascular response of crustaceans to low oxygen tensions.
and from a molecular prospective, how low oxygen in the environment alters oxygen binding pigment properties.

Cardiovascular compensation

The complexity of the open circulatory system of crustaceans has been somewhat underestimated. While some animals within Crustacea have crude circulatory systems, others like decapod crustaceans have a very complex system consisting of a single ventricle with multiple inflow (ostia) and outflow (arterial) valves. A set of complex neuro-hormonal mechanisms regulate both the heart and the valves thereby regulating flow throughout the cardiovascular system (McMahon, 1999; Maynard, 1960). A cardiovascular response to hypoxia may include alterations in cardiac output \( (V_b) \) via stroke volume \( (V_s) \) and heart rate \( (f_h) \), and alterations in hemolymph flow (McMahon, 2001). At a biochemical level, hypoxia may alter oxygen binding pigment affinity and/or concentration (Hochachka and Lutz, 2001). Overall these mechanisms serve to enhance \( O_2 \) conductance during hypoxic exposure.

**Cardiac parameters: heart rate, stroke volume and cardiac output**

Exposure to a hypoxic environment induces bradycardia in many decapod crustaceans as demonstrated in lobster (Reiber 1998), crayfish (deFur and Magnum, 1979; Wheatly and Taylor, 1981; Reiber, 1995; Reiber and McMahon, 1998) and crabs (Airriess and McMahon, 1994). However, this does not necessarily lead to a decrease in cardiac output. In crayfish and crabs, cardiac output is maintained despite the fall in heart rate via an increase in stroke volume.
The decrease in heart rate lengthens the amount of time in diastole allowing additional filling time for the ventricle and enhancing stroke volume (Reiber, 1995). In the mysid *Gnathophausia ingens* exposed to severe hypoxia, heart rate may fall more than 50%, from 110 bts min-1 in normoxia to 42 bts min-1 in extreme hypoxia yet this hypoxia tolerant animal is able to increase cardiac output, presumably via large increases in stroke volume (Belman and Childress, 1976). In lobster, there is a gradual decrease in heart rate during hypoxic exposure that becomes significant when reaching a PO2 of 30mmHg without any significant change in stroke volume (Reiber and McMahon, 1998). In this case the alterations in cardiac output mirror the changes in heart rate.

While hypoxia induced bradycardia is considered the typical response, it is not true for all crustaceans. There exist several examples of hypoxia induced tachycardia as observed in the cladoceran *Daphnia magna* (Paul et al., 1998; Pirow et al., 1999), in larval and small juvenile shrimp *Metapenaeus enisis* (McMahon et al., 1995) and in small adult grass shrimp *Palemonetes pugio* (Harper and Reiber, 1999). In these examples cardiac output is increased or maintained primarily due to a sufficient increase in rate of pumping even in the face of a decreased filling volume. Whether heart rate increases or decreases during hypoxic exposure the animal, attempts to maintain cardiac output and \( O_2 \) convection. If hypoxic conditions become severe the animal may reach its \( P_{\text{crit}} \) and no longer be able to maintain cardiac output and aerobic metabolism.
Cardiac parameters and reproduction

Life history stage, and specifically reproductive state, can have significant effects on an animal's physiological responses to perturbations in the environment. During reproduction, energy allocation and energy requirements (reflected by oxygen uptake and cardiac function) change due to the metabolic cost associated with gamete production and/or active brood care (Harrison, 1990; Vernberg & Piyatiratitivorakul, 1998; Naylor & Taylor, 1999; Baeza & Fernandez, 2002). The production of gametes via vitellogenesis (Bauer & Abdalla, 2000) represents an energy-demanding metabolic event for all gravid females (Chang & O'Connor, 1983; Adiyodi, 1985). However, energy demands following gamete production can vary depending on the extent of parental care. Groups that directly disperse fertilized eggs into the environment (e.g. euphausids, penaeids) incur a low metabolic cost while groups that actively care for embryos and/or offspring (e.g. amphipods, isopods) incur greater metabolic costs (Baeza & Fernandez, 2002). Much like crabs, female shrimp, belonging to the Family Palaemonidae, do not care for their offspring after hatching; however, they do provide care for the embryonic brood attached to their abdomen. Active brood care among crabs is associated with an increase in oxygen consumption by ovigerous females (Fernandez, Bock & Portner, 2000). The metabolic cost of brood care has not been well studied and it may impact the physiological ability of reproducing females to respond to acute changes in their external environment.
While animals may respond effectively to the demands of hypoxic stress or reproduction independently, the energetic demands of reproduction are often superimposed on the physiological stresses associated with environmental hypoxia. In crustaceans, acute hypoxic exposure has been shown to increase female brooding behaviours to enhance oxygen supply to embryos (Wiklund & Sundelin 2001). Unfortunately, investigations of the physiological responses of reproducing females to acute hypoxic exposure are lacking in the literature. Additionally, while considerable attention has been given to brood care in brachyurans (crabs) little data exists on brood care and responses of ovigerous and/or gravid females in macrurans (shrimp, lobster, crayfish).

**Hemolymph flow**

Numerous studies show that decapod crustaceans can redistribute hemolymph flow among their arterial systems, which include the anterior aorta (AA), the paired anterior lateral arteries (ALA), the paired hepatic arteries (HA), the posterior aorta (PA) and the sternal artery (SA) (Fig. 1). The redistribution of arterial hemolymph flow is accomplished by muscular cardio-arterial valves located where individual vessels exit the heart (Maynard, 1960). The ability to redistribute hemolymph flow through these valves has been demonstrated for several species (*Bathynomus* Kihara and Kuwasawa, 1984; *Cancer magister* McGaw et al., 1994; *Panulirus japonicus* Kuramoto and Ebara, 1984a; *Procambarus clarkii* Reiber, 1994). Beyond the cardio-arterial valves, flow and resistance can be altered in the PA due to muscle bands in the lateral wall of this vessel and due to valves located between the PA and the branching segmental
lateral vessels (Wilkens, Davidson and Cavey, 1997; Wilkens and Taylor, 2003). Otherwise, there are no muscles in the walls of decapod arteries to allow for changes in vascular resistance (Shadwick, Pollock and Strieker, 1990).

Changes in flow during hypoxia have been demonstrated and, in general, when oxygen is limited, most animals redirect flow away from other vessels in favor of flow the SA (Reiber and McMahon, 1998). The SA exits the heart ventrally and divides into a small posterior and larger anterior branch. Both the anterior portion and posterior portion supply the ventrally located ventral nerve cord along most of the length of the animal. Additionally, the anterior portion supplies most of the walking legs and the buccal apparatus, including the scaphognathites (McMahon et al., 1997). In this manner hemolymph flow is redirected toward in favor of the nervous tissue and since the anterior portion of the SA supplies the scaphognathites also assists in providing sufficient O$_2$ to maintain ventilation. Alterations in hemolymph flow have not been studied in reproductively active females whose flow patterns may be altered significantly from the patterns observed in non-reproducing animals.

**Pressure-area relationships**

The pressure-volume (P-V) relationship has been used extensively to study the mechanics of the cardiac cycle in the closed circulatory systems of mammalian and avian systems yet this valuable tool has not yet been applied to the study of cardiac dynamics in open systems. The "classic" P-V loop (Fig. 2) provides data on cardiac dynamics that cannot be gained from independent measures of pressure and volume. The cardiac cycle consists of four distinct
phases: (1) iso-volmic contraction (2) ventricular emptying (3) iso-volmic relaxation (4) ventricular filling). The area enclosed by the loop during one cardiac cycle is a measure of stroke work (Berne and Levy, 1986) and is correlated to myocardial O\textsubscript{2} consumption (Sagawa et al., 1988). Cardiac work is the product of stroke work and heart rate to provide an estimate of ventricular work over time, in this case per minute.

The ability to measure pressure within the ventricle coupled with the ability to measure area, digitally and in vivo, allows for a relatively non-invasive method for the determination of cardiac dynamics. Simultaneous recording of intracardiac pressure and, using automated boarder detection, the area of the chamber, results in the ability to generate pressure area (P-A) loops. P-A loops provide comparable data to P-V loops and due to the ease of obtaining area data are currently used in both laboratory and clinical settings (Keller et al, 1991; Senzaki, 2001). Since P-A loops have not been used in the assessment of ventricular dynamics in an open circulatory system, the use of P-A loops will provide additional, key data for elucidating the cardiac mechanics and energetics involved in the hypoxic response.

**Molecular compensation of oxygen binding pigments**

There are two major types of oxygen binding proteins that reversibly bind oxygen, those with iron (hemoglobin and hemorythrin) and those with copper (hemocyanin) (Terwilliger, 1998). All three can be found in invertebrate phyla. Extracellular hemocyanin (Hc) is the primary oxygen binding pigment of decapod
crustaceans. The active site of the Hc molecule contains six conserved histadines that bind the two copper atoms required to bind a single oxygen molecule. In crabs the extracellular Hc molecule has six polypeptide chains that assemble into one and two hexamers. Three of the polypeptide chains are invariable and the other three are highly variable (Mangum and Rainer, 1988). The Hc of normoxic crabs contains all six chains and has a low oxygen binding affinity; however upon exposure to hypoxia, hypoxic animals have reduced levels of the variable chains and progressively higher oxygen binding affinities. The Hc of hypoxic crabs contained one hexamers composed of invariant chains while normoxic crabs contains two hexamers composed of both variant and invariant chains (Mangum et al., 1991).

The branchiopod crustaceans use large extracellular Hb as their oxygen binding molecule. Hemoglobin structure varies widely among branchiopods. The Hbs are diverse in their quaternary structure and subunit size, however they share the same myoglobin fold and heme moiety as red blood cell Hb (Terwilliger, 1998). The Hbs are large extra-cellular molecules ranging from 220 kDa in Artemia salina (Moens and Kondo, 1978) to nearly 800 kDa in Lepidurus apus lubbocki (Ilan and Daniel, 1979) and is composed of various sized subunits. Among the Cladocera and Notostraca, Hb subunit chains range from 30-37 kDa (Peeters et al., 1990), with two heme groups per chain (Ilan and Daniel, 1979). In the Notostracans Lepidurus apus lubbocki and Lepidurus bilobatus, the native Hb molecules have molecular weights of approximately 798 kDa and 680 kDa, respectively, with subunits in the 33 – 34 kDa range (Dangott and Terwilliger,
Triops longicaudatus Hb has a molecular weight of ~600 kDa, and Horne and Beyenbach (1974) estimated the molecular weight of the subunits at approximately 20.5 kDa.

During periods of chronic hypoxia certain branchiopods such as *Daphnia magna* (Fox, 1955; Zeis, 2003), *A. salina* (Gilchrist, 1954; Heip et al, 1978) and *T. longicaudatus* (Scholnick and Snyder, 1996; Harper, 2003) increase Hb content; this response has been observed in both experimental and natural populations (Kobayashi and Hoshi, 1982; deWachter et al., 1992). Moreover, branchiopods modify Hb structure and functional properties in response to hypoxia (Wolf et al., 1983; Zeis et al., 2003). In *D. magna* and *T. longicaudatus*, hypoxia induces an increased Hb oxygen-binding affinity (Wolf et al., 1983; Kobayashi et al., 1994; Harper, 2003; Zeis et al., 2003). The branchiopod hypoxic response may also include differential Hb subunit assembly (Kimura et al., 1999) and differential subunit expression, as demonstrated in *D. magna* (Zeis et al., 2003). *Triops longicaudatus* like other branchiopods is extremely hypoxia tolerant yet little is known about the developmental and hypoxia-dependent kinetics of Hb expression in Notostraca. With its short life-span (~ 30-40 days) and ease of rearing in laboratory conditions, the use of *T. longicaudatus* will provide a vital link to further our understanding between the molecular and physiological responses to hypoxia.
Hypotheses

Despite the available data on the adult cardio-respiratory responses to hypoxia the literature lacks sufficient detail on the response to hypoxic stress during differing life history stages, in particular the responses of reproductively active females to hypoxia. The objectives of the first two chapters of this dissertation are to elucidate the responses of reproductively active females to hypoxic stress.

There is a strong correlation between cardiac output and metabolic rate in mammals, fishes, amphibians and invertebrates. Cardiac output was used as an estimate of metabolic rate differences in three life history stages of the grass shrimp *Palemonetes pugio*: (1) non-gravid (2) gravid (egg in the ovary) (3) ovigerous (brooding eggs) and gravid. The following hypotheses are tested in Chapter 2:

H1: Females in advanced reproductive states would have a greater metabolic demand based on the measurement of cardiac output values under normal oxygen saturation.

H2: Females in advanced reproductive states would have a decreased hypoxia tolerance. This estimate will be based on changes in cardiac output that occur when females are exposed to various levels of hypoxia.

Even though cardiac output values from Chapter 2 fell 15-25%, animals were not stressed to the point that they could not recover from hypoxic stress. Animals were placed back into holding tanks and survived even after exposure to...
the most severe hypoxic stress. This led to the question how is cardiac output redistributed in reproducing females? Is there preferential perfusion of tissues that allows for survival? Changes in hemolymph flow have been documented in non-reproductive decapod crustaceans in response to hypoxia however the literature lacks data on alterations in hemolymph flow when reproductively active females are exposed to hypoxia. Given that most crustaceans redirect flow to neural tissues during hypoxic exposure, the following competing hypothesis are tested in Chapter 3:

**H0**: As reported in non-reproducing animals, gravid females would redirect flow to neural tissue.

**H1**: Gravid females would preferentially redirect flow to the ovaries and developing eggs.

The data from chapter 3 supports the hypothesis that gravid females, like other non-reproductively active individuals, preferentially redirect flow to neural tissues during times of hypoxic stress. The data thus far did not provide a concrete answer to the question of how grass shrimp survived even severe hypoxic exposure, at values below their $P_{\text{crit}}$. In Chapters 4 and 5, the use of P-A loops adds to our understanding of cardiac mechanics and energetics. This tool has not been used in the heart of an animal with an open circulatory system. Chapter 4 tests the hypothesis that P-A loops are able to define cardiac function from a single ventricle, open circulatory system and compare the features of the P-A loop to those of the “typical” loops obtained from ventricles of animals with closed circulatory systems. Since the use of P-A loops is unexplored in open
systems, these studies do not use reproductively active females. All females in Chapters 4 and 5 were non-gravid in an effort to establish initial baseline data.

After defining the features and value of using P-A loops in the single ventricle of *P. pugio*, Chapter 5 applies this tool to the study the hypoxic response in *P. pugio*. Intra-cardiac pressure and stroke work (as determined from P-A loop area) are both new variables not yet used in the study of the hypoxic response of *P. pugio* and these two variables were used to test the following competing hypotheses:

- H1a: Intra-cardiac pressure will not change with decreasing O2 tension.
- H1b: Intra-cardiac pressure will decrease with decreasing O2 tension.
- H1c: Intra-cardiac pressure will decrease with decreasing O2 tension but only after reaching a critical hypoxic challenge.
- H2a: stroke work will not change with decreasing O2 tension.
- H2b: stroke work will increase with decreasing O2 tension.
- H2c: stroke work will decrease with decreasing O2 tension.

Crustaceans can experience extremes of oxygen concentration throughout their lifespan and cardiovascular adjustments provide a short-term mechanism to match O2 supply and demand during times of hypoxic stress. Changes in oxygen binding pigment concentration and binding properties provide an additional source for increased efficiency of O2 transport during times of hypoxic stress.
The previously reported changes in *Triops longicuadatus* Hb binding properties led to the investigation of altered expression of Hb subunits during hypoxia. As a result Chapter 6 addresses the following hypotheses:

**H1**: That developmental hypoxia will induce a change in Hb subunit composition that can explain the observed differences in O$_2$ binding.

**H2**: That exposure to hypoxia in adults can induce a change in Hb subunit composition.

Using hypoxia to test these hypotheses further enhances our understanding of the suite of cardiovascular mechanisms available to organisms and their regulation under times of stress.
References


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(Palaemonetes pugio Holthius) from the north inlet estuary, South Carolina. 


Figure 1: Lateral view of shrimp showing the location of the five arterial systems, ovary, hepatopancreas and nervous tissue. (modified from McLaughlin, 1983)

Figure 2: Pressure-volume loop of the left ventricle for a single cardiac cycle. (Adapted from Berne and Levy, 1986)
Figure 2

All valves closed

Ventricular emptying

Intra-cardiac pressure Exceeds peripheral pressure

Iso-volumetric contraction

Iso-volumetric relaxation

Stroke volume

End-systolic volume

50 ml 120 ml

Ventricular volume (ml)

Ventricular emptying

0 50 100

Ventricular pressure (mmHg)

End-diastolic volume

50 ml

Ventricular volume (ml)

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CHAPTER 2

THE INFLUENCE OF REPRODUCTIVE STATE ON CARDIAC PARAMETERS AND HYPOXIA TOLERANCE IN THE GRASS SHRIMP, *Palaemonetes pugio*

This chapter has been published in Functional Ecology and is presented in the style of that journal. The completed citation is:


DOI: 10.1111/j.1365-2435.2005.01062.x
Summary

1. In many crustaceans, female reproduction represents a time of increased metabolic demand. *Palaemonetes pugio* are typically hypoxia tolerant; however, the energetic demands of reproduction may compromise their ability to tolerate hypoxic conditions. Given the correlation between cardiac output and metabolic demand, we used cardiac output (CO) to measure differences in metabolic demand in the life history stages of *P. pugio*.

2. We hypothesized that (1) the cost of egg production would result in an increased CO for gravid females compared to non-gravid females; (2) those females that were both ovigerous and gravid would have an additional metabolic demand due to brooding behaviour (pleopod fanning) and hence an even greater CO and (3) hypoxia tolerance would decrease with increasing reproductive demand. To test these hypotheses, we compared cardiac output, across three reproductive states and at decreasing water oxygen tensions.

3. Ovigerous females had significantly greater pleopod fanning frequency than non-ovigerous females at all oxygen tensions. Additionally, ovigerous/gravid females had significantly higher cardiac output at all oxygen tensions than gravid only or non-gravid females.

4. Changes in cardiac output indicate that females became more sensitive to environmental oxygen tension with increasing reproductive demand. Non-gravid females were able to maintain cardiac output down to 15 mmHg O₂.
whereas gravid and ovigerous/gravid females maintained cardiac output down to 50 mmHg and 75 mmHg O$_2$ respectively.

5. These differences in CO suggest that metabolic demands of females change with reproductive state and, while gravid and ovigerous/gravid females appear more sensitive to low oxygen tensions they are able to physiologically tolerate low environmental oxygen conditions.

*Key-Words:* brood care, cardiac performance, hypoxia, reproduction
Introduction

Reproduction is an energy demanding event consisting of both direct and indirect metabolic costs, which are often "paid" in the form of trade-offs in activity, growth or storage (Stearns, 1989). Direct costs include gamete production, while indirect costs may include courtship, mate and/or territorial defense and parental care during embryonic and/or larval stages (Clutton-Brock, 1991). Among ectotherms, the direct metabolic costs of egg production and the indirect metabolic costs associated with parental care are greater for females than males (Clutton-Brock, 1991). As a result, reproductively active females incur additional energetic demands on their "normal" non-reproductive physiology that may affect their ability to regulate other physiological processes in changing environments.

Environmental factors including but not limited to salinity, temperature, pH and oxygen tension influence the physiological state of marine crustaceans. Life history stage, and specifically reproductive state, can have significant effects on an animal's physiological responses to perturbations in the environment. During reproduction, energy allocation and energy requirements (reflected by oxygen uptake and cardiac function) change due to the metabolic cost associated with gamete production and/or active brood care (Harrison, 1990; Vernberg & Piyatiratitivorakul, 1998; Naylor & Taylor, 1999; Baeza & Fernandez, 2002). The production of gametes via vitellogenesis (Bauer & Abdalla, 2000) represents an energy-demanding metabolic event for all gravid females (Chang & O'Connor, 1983; Adiyodi, 1985). However, energy demands following gamete production can vary depending on the extent of parental care. Groups that directly disperse...
fertilized eggs into the environment (e.g. euphausids, penaeids) incur a low metabolic cost while groups that actively care for embryos and/or offspring (e.g. amphipods, isopods) incur greater metabolic costs (Baeza & Fernandez, 2002). Much like crabs, female shrimp, belonging to the Family *Palaemonidae*, do not care for their offspring after hatching; however, they do provide care for the embryonic brood attached to their abdomen. Active brood care among crabs is associated with an increase in oxygen consumption by ovigerous females (Fernandez, Bock & Portner, 2000). The metabolic cost of brood care has not been well studied and it may impact the physiological ability of reproducing females to respond to acute changes in their external environment.

Animals living in coastal waters, especially in estuaries, are often exposed to fluctuations in oxygen availability (McMahon, 1988). *P. pugio* are no exception in that they may be exposed to daily fluctuations in oxygen tension that range from 9 to 170 mmHg (Cochran & Burnett, 1996). Thus, when faced with low oxygen tensions they must be able to adjust to or tolerate hypoxic conditions. Physiological responses of crustaceans to hypoxic environments include alterations in ventilation, cardiac output, arterial flow and oxygen carrier performance (Wheatly, 1981; McMahon, 1988; Reiber, 1995; McMahon, 2001; Guadagnoli & Reiber, 2005). Cardiac parameters, such as heart rate, stroke volume, and ultimately cardiac output can be adjusted to increase oxygen conductance during progressive hypoxia (Reiber, 1995) allowing the cardiovascular system to match oxygen supply and demand. An increase in oxygen utilization via greater oxygen extraction from the hemolymph results in an
increase in cardiac output to meet the increased tissue demand (Webber, Boutilier & Kerr, 1998); therefore, cardiac output is highly correlated with the rate of oxygen uptake in invertebrates (Herried, 1980) and fishes (Webber et al., 1998).

While animals may respond effectively to the demands of hypoxic stress or reproduction independently, the energetic demands of reproduction are often superimposed on the physiological stresses associated with environmental hypoxia. In crustaceans, acute hypoxic exposure has been shown to increase female brooding behaviours to enhance oxygen supply to embryos (Wiklund & Sundelin 2001). Unfortunately, investigations of the physiological responses of reproducing females to acute hypoxic exposure are lacking in the literature. Additionally, while considerable attention has been given to brood care in brachyurans (crabs) little data exists on brood care and responses of ovigerous and/or gravid females in macrurans (shrimp, lobster, crayfish). The aims of the current study were (1) to investigate the differences in brooding behaviour between ovigerous and non-ovigerous females, (2) to establish differences in cardiac output, an indicator of metabolic rate, between reproductive groups and (3) to assess the interaction between the metabolic demands of reproduction (egg production and brood care) and the stress of acute hypoxic exposure in the grass shrimp, *Palaemonetes pugio*.
Materials and Methods

*Palaemonetes pugio* (grass shrimp) were purchased from Gulf Specimen Marine Laboratories Inc. (Panacea, FL, USA). Shrimp were maintained in aerated 20-l aquaria filled with artificial seawater (salinity = $32 \pm 2\%$) at $20 \pm 2^\circ$C and were fed twice a week (commercial fish flake food). Animals were maintained in laboratory conditions for at least two weeks prior to experimentation and all experiments were performed at the same salinity and temperature as that of the holding aquaria. Shrimp were not fed for 48 h prior to experimentation. Females were classified and divided into 3 groups: non-gravid (no evidence of ovarian development), gravid (ovaries well defined) and ovigerous/gravid (eggs on pleopods with the next brood developing in the ovary). Shrimp were viewed under a stereo microscope (Leica Stereo Zoom 6 Photo) to determine the level of ovarian development, which is readily apparent through the transparent exoskeleton of *P. pugio*.

Wet weight for all animals was obtained prior to experimental use. The mean mass of females in all experiments was $145.8 \pm 3.5$mg. (non-gravid: $142.0 \pm 4.0$; gravid: $145.7 \pm 4.1$; ovig/gravid: $148.4 \pm 7.5$). In the case of ovigerous/gravid females, eggs were removed from the pleopods after the experiments and females were re-weighed to determine the contribution of the egg mass to the total weight of the animal. Egg mass accounted for 15% of an ovigerous/gravid female's total body mass; therefore, the average mass of ovigerous/gravid females carrying eggs was $179 \pm 7.5$mg.
Brood care

Ovigerous females of *P. pugio* attach eggs to pleopods located on the ventral surface of the tail until hatching. They fan their pleopods to enhance water flow in and around the developing brood. To determine whether differences in pleopod fanning existed, females were separated into two groups: non-ovigerous and ovigerous. Groups were first compared under normoxic conditions. Briefly, 5 females from each group were placed in individual meshed containers in a 10-l aquarium with water PO$_2$ maintained between 135-150 mmHg. Visual observations of pleopod fanning frequency were counted manually every 30 min for a 3 hour period. The following morning females were exposed to hypoxia. Water oxygen tension was lowered every 30 minutes in a step-wise manner and observations were made at 150, 100, 75, 50 and 15 mmHg PO$_2$. Changes in fanning frequency occur after 5 min of hypoxic exposure and stabilize after 15 min, therefore to ensure steady state fanning frequency, pleopod fanning frequency was recorded at the end of each 30 min hypoxic period and the average of three one-minute observations was recorded.

Cardiac parameters

Cardiac output (CO), calculated from heart rate (HR) and stroke volume (SV), was used to evaluate differences in energy demand between the three groups under normoxic conditions and in response to changes in water PO$_2$. Using a minimal amount of cyanoacrylate glue, animals were attached by their lateral cephalothorax to a flattened end of an applicator stick. Shrimp were then placed in a flow-through experimental chamber (for details see Harper & Reiber,
1999) with a surrounding enclosure to minimize visual disturbance during experiments. Water $P_{O_2}$ of the experimental chamber was maintained using a gas mixing flow meter (Cameron Instruments, GF-3/MP) ($P_{O_2} = 150, 100, 75, 50$ and $15 \text{ mmHg } O_2$). A dissecting microscope (Leica Stereo Zoom 6 Photo) equipped with a video camera (World Precision Instruments, Inc. Oscar Color Camera Vidcam), super VHS video recording system and a Horita time code generator was used to record cardiac activity. Animals from each reproductive group were exposed to one level hypoxia as follows: Females were acclimated for 2 h under normoxic conditions ($150 \text{ mmHg } O_2$), and the heart recorded for 2 minutes. Females were then exposed to an experimental oxygen condition ($P_{O_2} = 100, 75, 50$ or $15 \text{ mmHg } O_2$) for one hour and cardiac changes were recorded for a further 2 min. Based on previous studies of cardiovascular parameters one hour is sufficient to reach steady state in $P. pugio$ (Guadagnoli & Reiber, 2005; Harper & Reiber 1999).

Cardiac parameters (HR and SV) were measured using video-microscopic dimensional analysis. Videotapes were advanced frame-by-frame using an editing tape player (Panasonic AG-DS550). Selected frames were captured using frame grabbing software (Snappy Video Snapshot, Play, Inc.) and imported into Scion Image for measurement. The heart was modeled as a trapezoid and cardiac volume was determined as follows: Cardiac volume = $w \left(0.5h(b + a)\right)$ 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 (Harper & 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. A minimum of 3 cardiac cycles was analyzed for each animal at each treatment.

**Statistical analysis**

A two-way RMANOVA (Sigma stat 3.2) was used to determine differences in fanning behaviour between groups with decreasing oxygen tensions. A mixed model ANOVA (SAS) as used to determine differences in cardiovascular parameters between groups and within groups at the varying oxygen tensions using a 3 (reproductive group) x 5 (oxygen tension) x n (number of females) design. Tukey tests were used to determine significant differences in O$_2$ tension among groups. Between 5 -10 females from each group were tested at each oxygen tension. All values are reported as means ± SE with (n) indicating the number of animals. Level of significance was set at p < 0.05.

**Results**

**Brood care**

A significant difference was found between ovigerous and non-ovigerous females in pleopod fanning behaviour ($F_{1, 18} = 484.89, p < 0.001 \ n = 20$), with fanning frequency greater in ovigerous females than non-ovigerous females at all oxygen tensions. During the three hour normoxic observation period, ovigerous shrimp fanned significantly more ($35 ± 2$ bts/min) than non-ovigerous shrimp ($<1± 0.9$ bt/min) (Fig. 1). Oxygen tension also had a significant effect on fanning frequency ($F_{4, 72} = 105.99, p < 0.001$; Fig 1), with frequency increasing at lower
PO$_2$ especially in the ovigerous shrimp. Non-ovigerous shrimp showed little or no fanning behaviours until reaching 15 mmHg O$_2$. In contrast, ovigerous shrimp fanned an average of 48.5 ± 3.5 bts/min with a significant increase in fanning frequency at 50 mmHg O$_2$, reaching a peak of 135 ± 5.8 bts/min at 15 mmHg O$_2$ (Fig 1). While not measured directly, we observed an increase in the amplitude of ovigerous pleopod fanning when oxygen tension fell to 50 mmHg O$_2$ and 15 mmHg O$_2$.

**Cardiac parameters**

Heart rate differed significantly between reproductive groups ($F_{2,85} = 5.2, p < 0.001$) as well as within each group for declining oxygen tension ($F_{4,76} = 31.78, p < 0.001$). There was also a significant interaction between reproductive group and oxygen tension ($F_{8,76} = 2.65, p = 0.013$; Fig. 2a). Stroke volume differed significantly between reproductive groups ($F_{2,85} = 10.47, p < 0.001$), with ovigerous/gravid females having the highest values; however, there were no significant differences in stroke volume within groups with declining oxygen tension ($F_{4,76} = 2.42, p = 0.06$; Fig. 2b)

Cardiac output values of the non-gravid group (36.67 ± 2.4 µl/min, $n = 22$) during normoxia were the lowest of all groups, representing a "baseline" level of metabolic demand. Gravid females had a 13.8% greater cardiac output (42.48 ± 2.2 µl/min, $n = 36$) and ovigerous/gravid females had a 34.3% greater cardiac output (55.83 ± 2.5µl/min, $n = 30$) than non-gravid females ($F_{2,85} = 13.23; p < 0.001$; Fig 2c) under normoxic conditions. Cardiac output was also significantly different within groups with declining oxygen tension ($F_{4,76} = 31.78, p < 0.001$),
Within each treatment and reproductive group the change in heart rate contributes to the significant changes in cardiac output. Overall, ovigerous/gravid females had the highest values for all cardiac parameters at all oxygen tensions, while non-gravid females had the lowest.

Non-gravid females were most hypoxia tolerant, with cardiac output values varying less than 5% until 15 mmHg at which point cardiac output fell significantly ($p = 0.015$; Fig 3). Gravid females were able to maintain cardiac output within 5% of normoxic values until reaching 50 and 15 mmHg, when cardiac output dropped 18% and 15% respectively ($p = 0.03$; Fig 3). Ovigerous/gravid females were least hypoxia tolerant with a >15% decline in cardiac output beginning at 75 mmHg ($p < 0.001$; Fig. 3).

Discussion

Recently, there has been renewed interest in the metabolic demand of parental care, in particular brood care, in marine invertebrates. We have established that ovigerous $P. pugio$ exhibit brooding behaviour in the form of pleopod fanning that is enhanced during times of hypoxic stress. The observed increase in cardiac output with advanced reproductive states (gravid and ovigerous/gravid) supports the idea that females incur greater metabolic demands than non-gravid females due to egg production and/or brooding behaviour. Although gravid and ovigerous/gravid females were more sensitive to declining $P_0_2$, the lack of interaction between reproductive state and $P_0_2$ indicates that reproductively active females of $P. pugio$ are well adapted to

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hypoxic stress and respond to hypoxic stress in a manner similar to non-gravid females.

Little or no pleopod fanning was observed in non-ovigerous females of \( P. pugio \), while ovigerous females fanned their pleopods (and broods) even during normoxic conditions and continuously increased fanning frequency as water oxygen tension was lowered. Increased brood fanning with decreased oxygen tension has been reported in amphipods (Dick, Fallon & Elwood, 1998; Dick, Bailey & Elwood 2002), crayfish (Ameyaw-Akumfi, 1976) and crabs (Naylor, Taylor & Bennett, 1997; Baeza & Fernandez, 2002; Brante et al., 2003; Fernandez et al., 2003). Oxygen limitation in the center of the egg mass has been documented for crabs & brood behaviour has been hypothesized to enhance oxygen delivery to the center of egg masses (Naylor et al., 1997; Fernandez et al., 2000, 2003). In \( C. maenas \) active brood care enhances the \( P_{o2} \) of the egg mass by as much as 10mmHg above ambient water \( P_{o2} \) at 45 mmHg (Wheatly, 1981). While this is beneficial and necessary for the brood, our study suggests that brooding behaviour places an additional metabolic demand on ovigerous females.

The energetic cost of brood care includes both egg-bearing (Taylor & Leelapiyanart, 2001) and brooding behaviour (Fernandez et al., 2000; Taylor & Leelapiyanart, 2001; Baeza & Fernandez, 2002; Brante et al., 2003). In \( P. pugio \), 15% of an ovigerous female’s mass was accounted for by the developing brood, similar to previous findings in the crab species \( Heterozius rotundifrons \) and \( Cyclograpsus lavauxi \) in which egg clutch mass contributed to 11.3% and 17.2%.
of female mass respectively (Taylor & Leelapiyanart, 2001). In both crab species females carrying early embryos in showed a slight increase in mass specific rates of oxygen consumption compared to their non-ovigerous counterparts, a difference attributed to egg-bearing alone (Taylor & Leelapiyanart, 2001). In the current study, under normoxic conditions, gravid *P. pugio* females had a 13.8% higher cardiac output than their non-gravid counterparts, a difference that could be attributed to the increased metabolic demand of vitellogenesis. Females that were ovigerous/gravid had a 25% greater cardiac output than gravid females and 34.3% greater cardiac output than non-gravid females (Fig. 2c), suggesting that there is an increased energy demand associated with both egg bearing and brooding. Overall, the differences in cardiac output across groups is indicative of the difference in metabolic energy demands between different life history stages.

In *P. pugio* cardiac output values increase with advanced reproductive states. During the process of vitellogenesis and oogenesis females have increased metabolic demand due to the increased lipid metabolism necessary for these processes. This may be the result of additional energy allocation to both vitellogenesis and brood care. Energy allocation studies of *P. pugio* have shown that 51.7% of total energy expenditure can be attributed to reproductive energy and 25.4% to respiration (Vernberg & Piyatiratitivorakul, 1998); therefore, a 34.3% increase in cardiac output above "baseline" is not surprising. In the present study there was no interaction between reproductive state and oxygen tension, suggesting that all three groups responded similarly to hypoxic stress.
The significant drop in cardiac output for non-gravid females seen at 15 mmHg but not 50 mmHg corresponds well to previously reported $P_{\text{crit}}$ values for *P. pugio* (~35 Torr from respirometry; Cochran & Burnett, 1996 and ~ 40 Torr from cardiac output estimates; Harper & Reiber, 1999). The respirometry estimates of $P_{\text{crit}}$ at 53 Torr (Guadagnoli & Reiber, 2005) for gravid females corresponds well to the fall in cardiac output for gravid females in this study. Respirometry measurements of $P_{\text{crit}}$ for ovigerous/gravid females are not currently available but the estimated value based on cardiac output may be a close estimate.

On the other hand, even though cardiac output in the ovigerous/gravid group drops 15% at 75 mmHg $O_2$ this value may not be biologically relevant or sufficient to conclude that animals are unable to sustain aerobic metabolism. The additional metabolic demand of reproduction may prove to be a greater challenge during prolonged periods of hypoxia or below 10 mmHg $O_2$. The current results suggest that other physiological factors may play a role in allowing ovigerous/gravid females to continue to increase pleopod fanning in the face of declining oxygen tension. Both non-gravid and gravid females redirect hemolymph flow upon exposure to hypoxia (Guadagnoli & Reiber, 2005) so that although cardiac output is declining, the animal has the ability to alter flow to metabolically active tissue at the expense of less active metabolic tissue. Other factors may include changes in hemocyanin concentration or oxygen binding affinity. During ontogeny the type and affinity of hemocyanin changes (for review see Terwilliger & Ryan, 2001) but changes in hemocyanin composition through adult reproductive stages remains unknown. Previous studies in crabs have
demonstrated that both neuro-hormonal factors (Morris & McMahon, 1989) and lactate (i.e. Trouchot, 1980, Saunders et al., 1992) circulating in the hemolymph increase hemocyanin binding affinity.

For species requiring any form of active brood care or post hatching care, the metabolic demands of reproduction continue after spawning. Much of embryonic brood care involves supplying oxygen to the embryos, for which ovigerous females incur a metabolic cost. This study demonstrates that *P. pugio*, like its crab counterparts; incurs a metabolic cost for egg formation as well as active brood care. The increased sensitivity of gravid and ovigerous/gravid *P. pugio* to hypoxic stress emphasizes the additional metabolic demands of reproduction. Given that cardiac output of gravid and ovigerous/gravid females drops only 15-20% even at 15 mmHg, it appears that maintenance of oxygen delivery in reproducing females is sustained by additional physiological processes which merit further investigation.

Acknowledgements

The authors would like to thank Dr. Steven Roberts and Dr. Iain McGaw for their critical review of the manuscript and Dr. Cheryl Vanier for her help with the statistical analysis. This research was funded by NSF grant to CLR and GSA grant to JAG.
References


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Figure legends

Figure 1: Differences in fanning frequency between ovigerous and non ovigerous females during normoxia and hypoxia. Fanning frequency was significantly greater in the ovigerous group than non-ovigerous group during the normoxic control trials (open symbols). Fanning frequency increased significantly with declining PO$_2$ (closed symbols) especially in the ovigerous group. (*) indicates significantly different from 150 mmHg, $p < 0.001$

Figure 2: Changes in cardiovascular parameters between reproductive groups with declining PO$_2$ (a) heart rate (b) stroke volume (c) cardiac output. ($) indicates the group (the line) is significantly different from non-gravid group, $p < 0.001$. (*) indicates a value significantly different from normoxia within a group, $p < 0.001$

Figure 3: Within group pair-wise comparisons of mean percent change in cardiac output. Non-gravid females were most hypoxia tolerant while non-gravid females were least hypoxia tolerant. (*) indicates a significant decrease from normoxic values.
Figure 1

![Graph showing the effect of hypoxia on fanning frequency over time. The graph includes lines for ovig control, ovig hypoxia, non control, and non hypoxia. The y-axis represents fanning frequency (bfs per min), and the x-axis represents time (min). The graph shows increasing fanning frequency with increasing hypoxic stress, with distinct marker points at different hypoxic pressures.]
CHAPTER 3

CHANGES IN CARDIAC OUTPUT AND HEMOLYMPH FLOW
DURING HYPOXIC EXPOSURE IN THE GRAVID GRASS
SHRIMP, PALAEMONETES PUGIO

This chapter has been published in the Journal of Comparative Physiology and is presented in the style of that journal. The completed citation is:


DOI: 10.1007/s00360-005-0487-z

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Abstract

The cardiovascular response of decapod crustaceans to hypoxic exposure is well documented; however, information is limited concerning the influence of reproductive state on cardiovascular demands during hypoxic exposure. Given the additional metabolic demand of reproduction we investigated the cardiovascular adjustments employed by gravid grass shrimp *Palaemonetes pugio* to maintain oxygen delivery during hypoxic stress. Cardiac output values were elevated in gravid compared to non-gravid grass shrimp. Gravid grass shrimp were exposed hypoxia and stroke volume, heart rate, cardiac output and hemolymph flow were determined using video-microscopy and dimensional analysis. Oxygen consumption rates were determined using respirometry. There were no changes in cardiac output values of gravid females until reaching 6.8 kPa O$_2$, with a significant redistribution of hemolymph flow at 13.7 kPa O$_2$. Flow was significantly decreased to the anterior lateral arteries that supply the ovaries and hepatopancreas, the anterior aorta and the posterior aorta. The redistribution of hemolymph flow away these vessels results in enhanced hemolymph flow to the sternal artery that supplies the ventral segmental system, the gills, the buccal apparatus and the ventral nerve cord. The data suggest that during hypoxic stress gravid females place a priority on survival.
Introduction

Cardiovascular responses to hypoxia vary among species depending on the mechanism employed to maintain oxygen delivery to the tissues. Cardio-respiratory responses to hypoxia include, but are not limited to, changes in heart rate, stroke volume, hemolymph flow, ventilatory rate and changes in $O_2$ binding properties of respiratory pigments (McMahon, 2001; Reiber and McMahon, 1998; Wheatly and Taylor, 1981). A few crustaceans exhibit a hypoxia-induced tachycardia such as the cladoceran *Daphnia magna* (Paul et al., 1998), the juvenile shrimp *Metapenaeus ensis* (McMahon et al., 2002), and the grass shrimp *Palaemonetes pugio* (Harper and Reiber, 1999). More typically, however, crayfish, crabs and lobsters, exhibit a hypoxia-induced bradycardia with a concomitant increase in stroke volume (McMahon, Burggren and Wilkens, 1974; Reiber, McMahon and Burggren, 1992; Reiber, 1995; Reiber and McMahon, 1998; Wheatly and Taylor, 1981). The increase in stroke volume may result from an increased filling time and allows the animal to maintain or even increase cardiac output compared to normoxic values.

As oxygen tension in the environment is reduced, eventually the animal reaches a critical oxygen tension ($P_{crit}$) at which point oxygen delivery is no longer sufficient to meet aerobic metabolic demand (Herreid, 1980; Reiber, 1995; Reiber and McMahon, 1998). Critical oxygen tension is correlated with cardiac output and may vary with environmental condition as well as physiological state (Herried, 1980). Oogenesis and vitellogenesis are energetically costly processes (Tsukimura, 2001) and increase the metabolic requirements of gravid females.
Even in this physiological state it remains important for the gravid female to secure oxygen delivery to all metabolizing tissues during hypoxic exposure.

Numerous studies show that decapod crustaceans can redistribute hemolymph flow among their arterial systems, which include the anterior aorta (AA), the paired anterior lateral arteries (ALA), the paired hepatic arteries (HA), the posterior aorta (PA) and the sternal artery (SA) (Fig. 1). The redistribution of arterial hemolymph flow is accomplished by muscular cardio-arterial valves located where individual vessels exit the heart (Maynard, 1960). The ability to redistribute hemolymph flow through these valves has been demonstrated for several species (Bathynomus Kihara and Kuwasawa, 1984; Cancer magister McGaw et al., 1994; Panulirus japonicus Kuramoto and Ebara, 1984a; Procambarus clarkii Reiber, 1994). Beyond the cardio-arterial valves, flow and resistance can be altered in the PA due to muscle bands in the lateral wall of this vessel and due to valves located between the PA and the branching segmental lateral vessels (Wilkens, Davidson and Cavey, 1997; Wilkens and Taylor, 2003). Otherwise, there are no muscles in the walls of decapod arteries to allow for changes in vascular resistance (Shadwick, Pollock and Strieker, 1990).

Most crustaceans redirect flow away from other vessels in favor of flow to the SA during hypoxia (Airriess and McMahon, 1994; McGaw, et al., 1994; Reiber and McMahon, 1998). The SA exits the heart ventrally and divides into a small posterior and larger anterior branch. Both the anterior portion and posterior portion supply the ventrally located ventral nerve cord along most of the length of
the animal. Additionally, the anterior portion supplies most of the walking legs and the buccal apparatus, including the scaphognathites (McMahon et al., 1997).

Taking advantage of the correlation between oxygen uptake and cardiac output, (Herried, 1980) we compared cardiac output between gravid and non-gravid females as a measure of the differences in metabolic demand between non-gravid and gravid shrimp. Given that grass shrimp from temperate climates can be successive breeders from February thru October (Bauer and Abdalla, 2000) most females, under our laboratory conditions, remain gravid throughout the year. *P. pugio* are caridean shrimp and therefore, after vitellogenesis is complete, the eggs are spawned and the embryonic brood is attached to pleopods located on the abdomen. While the effects of hypoxia on the developing brood has been studied, the effects of hypoxia on the gravid female is lacking in the literature. Therefore, we chose to examine changes in hemolymph flow during hypoxic exposure in gravid grass shrimp. Grass shrimp are typically hypoxia tolerant with $P_{\text{crit}}$ values ranging from 4.7 kPa (~35Torr) to 5.3 (~40 Torr) (Cochran and Burnett, 1996; Harper and Reiber, 1999), however we hypothesized that with the increased metabolic demand of reproduction, gravid grass shrimp would be not be able to maintain cardiac output at the same levels as non-gravid females and would have higher $P_{\text{crit}}$ values than those previously reported. Additionally, they should have greater flow to the ALA’s that supply the ovaries and hepatopancreas, but under hypoxic stress flow would necessarily be directed toward the SA to supply the ventral segmental system, the gills and the ventral nerve cord.
Materials and Methods

Grass shrimp, *Palaemonetes pugio*, were purchased from GulfSpecimen Marine Laboratories, Inc., (Panacea, FL) and maintained in 20L aquaria in aerated seawater (30-32 °/oo) at 20°C. Animals were maintained in laboratory conditions for two weeks prior to experimental use. Animals were fed marine flakes (Tetra) three times a week, with the exception of experimental animals which were separated from the general population and fasted two days prior to use.

The ovaries of gravid females are clearly visible through the transparent carapace. Gravid state was determined as follows (from Bauer and Abdalla, 2000): stage 1, no ovarian development, stage 2, ovary small, not extended above the cardiac stomach towards the anterior cephalothorax, stage 3, ovary large, extending into the space above the cardiac stomach, but not filling more than half of the anterior space, stage 4, ovary large and full, occupying most of the space above the cardiac stomach and filling the anterior space. Stage 1 animals were used as non-gravid and stage 3 and 4 were used in the gravid group. Stage 2 animals were not used in any experiments.

Grass shrimp (105 ± 5 mg) were attached to the flattened end of a wooden applicator stick on the lateral cephalothorax with cyanoacrylate glue. Animals were held in place and positioned within the experimental chamber with a micromanipulator (World Precision Instruments). Cardiac parameters were recorded through the transparent carapace with a stereo-microscope (Leica MZ 12.5, McBain Instruments) equipped with a video camera (World Precision Instruments).
Instruments), super VHS recorder (Panasonic HR-S4500U) and a Horita time code generator (VG50).

**Experimental design**

Water within the flow-through experimental chamber was maintained at 20°C and water oxygen partial pressure ($P_{O_2}$) was established and maintained using a gas mixing system (Cameron Instruments). For comparison of cardiac output between gravid and non-gravid females, an individual animal was placed in the experimental chamber in normoxic water (20.5 kPa $P_{O_2}$). After a two hour acclimation period the animals were video-taped for later analysis of cardiac parameters.

For the hemolymph flow experiments, gravid grass shrimp were exposed placed in normoxic water, acclimated for two hours and then water $P_{O_2}$ was lowered to one of three hypoxic levels; 13.7 kPa, 10.3 kPa or 6.8 kPa $O_2$ for one hour. Each animal was exposed to only one level of hypoxia. Cardiac parameters were recorded during normoxia and hypoxia and then the animal was injected for determination of hemolymph flow.

**Determination of cardiovascular parameters:**

**Heart rate, stroke volume and cardiac output**

Cardiac parameters, heart rate ($f_h$), stroke volume ($V_s$) and cardiac output ($V_b$) were measured using video-microscopic dimensional analysis. Videotape was advanced frame-by-frame using an editing tape player (Panasonic AG-DS550). As previously described by Harper and Reiber (1999), selected frames were captured using a frame grabbing software (Snappy Video Snapshot, Play,
Inc.) to determine cardiac volumes at end-systole (minimal dimension of the heart after contraction) and end-diastole (maximal dimension of the heart after filling). The heart was modeled as a trapezoid and cardiac volume was determined using the following equations: Cardiac volume = \( w \cdot 0.5h(b + a) \) 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 (Harper and Reiber, 1999). Stroke volume was calculated as the difference between cardiac volumes (end systolic volume - end diastolic volume) and \( V_b \) was calculated as the product of \( f_s \) and \( V_s \). A minimum of 3 cardiac cycles was analyzed for each animal at each treatment. A t-test was used to determine differences in \( f_s \), \( V_s \) and \( V_b \) between non-gravid and gravid females under normoxic conditions. Within the gravid group, the effects of hypoxia were compared at each level of hypoxia for each parameter (\( f_s \), \( V_s \) and \( V_b \)) using a paired t-test (Sigma Stat 2.0). Data are given as means ± SE with (\( n \)) indicating the number of animals.

**Respirometry**

Oxygen uptake and critical \( P_{O_2} \) were determined by measuring the depletion of oxygen in a closed system respirometer. Oxygen partial pressures were measured using a Cameron instruments oxygen meter with a Clark oxygen electrode. Output from the oxygen meter was recorded every 30 sec using DATAQ data acquisition system. A gravid female was placed into a 5 ml chamber containing well-aerated and filtered sea water at \( \sim 20.5 \) kPa and then the chamber was sealed. Experiments were terminated when the animal could
no longer maintain an upright position within the chamber. At the end of the experiment the exact volume of water in the chamber, the temperature of the water, and the weight of the animal were determined to calculate the rate of oxygen uptake per gram.

Critical oxygen pressure was calculated for each animal. The value for $P_{\text{crit}}$ was determined by calculating regression lines for the two portions of the oxygen uptake curve, a horizontal portion, where oxygen uptake is maintained and the steeply sloped portion when oxygen uptake was no longer maintained. The intersection of these two lines was designated as $P_{\text{crit}}$.

Comparison of sum of arterial flow using dye injection with video-microscopic dimensional analysis for the determination of cardiac output

Cardiac output can also be determined as the sum of the arterial flows of all the vessels leaving the heart. To test the accuracy this method, we compared $V_b$ determined by video-microscopy dimensional analysis (Harper and Reiber, 1999) with $V_b$ calculated from the sum of arterial flows. A dorsal view was used to obtain flow measurements within the AA, ALA, PA ($n=10$). A separate group of animals was placed in the experimental chamber and rotated $90^\circ$ to obtain a lateral view through the carapace for flow measurements within the SA and HA ($n=7$). Cardiac output was calculated as the sum of flows from the AA, ALA ($x2$), PA, HA ($x2$), and SA.

Determination of arterial hemolymph flow

The general anatomy and five major arterial systems of the grass shrimp are shown in Figure 1. Hemolymph flow in gravid females was calculated by
determining the velocity of a dye front through an artery and its vessel diameter. Images of vessels were captured on video and vessel diameters were determined as the mean of three measurements of internal vessel diameter. To measure movement of the dye front in the arteries, two frames were captured and analyzed to calculate the difference between the location of the dye front at time 1 and time 2. With this information flow was then calculated using the following equations: Velocity: \( v = \frac{\Delta d}{\Delta t} \); where \( \Delta d \) = change in distance (mm) and \( \Delta t \) = change in time (s). Flow: \( F = v \times A \); where \( v \) = velocity and \( A \) = cross-sectional area determined by \( \pi r^2 \) (\( r \) = radius). This technique allowed for a relatively non-invasive assessment of the vasculature, and measurement of the dye front was easily achieved through the transparent exoskeleton of the animal. Flow to the SA was estimated as the difference between \( V_b \) from dimensional analysis and the sum of flows from the AA, ALA (x2) and PA. As in previous studies with macrurans (Reiber et al., 1997), we found flow through the HA to be negligible due to their small diameter, therefore the hepatics were not considered in any further calculations.

Dye injection was achieved by using a pulled glass micro-pipette (tip diameter: 5–10 \( \mu \)m) filled with Evan’s blue dye in grass shrimp saline (600mMol NaCl, 10mMol CaSO_{4}, 10mMol MgSO_{4}, 5mMol KCl, 5mMol NaHCO_{3}) attached to a nanoliter injector (WPI, Sarasota, FL). The micro-pipette tip was inserted through the soft dorsal arthrodial membrane at the junction of the thorax and abdomen and slowly advanced into the ventricle. In a preliminary study, animals were acclimated with and without the micropipette in their ventricle and after two
hours there was no statistical difference between the two groups in $V_b$ (without pipette $V_b = 42.2 \pm 16$ ml/min; with pipette $V_b = 41.7 \pm 19$ul/min, $n = 10$, $p=0.85$); therefore all data were collected two hours after insertion of the micro-pipette. After two hours under normoxic conditions the heart was recorded on videotape for later analysis of $f$, $V_s$ and $V_b$. Animals in the normoxic control group were then injected with the dye solution and the movement of the dye front through the arterial system was recorded on videotape for 1 min prior to injection and up to 5 min post injection, for later calculation of dye front velocity and vessel diameter. The hearts of hypoxic treatment groups were recorded on videotape after exposing the shrimp to normoxic conditions for two hours to determine cardiac parameters only, then water $P_{O_2}$ was lowered to either 13.7 kPa, 10.3 kPa or 6.8 kPa for one hour. After one hour of hypoxic exposure the dye solution was then injected and movement of the dye front was recorded for later calculation of flow. To minimize any changes in cardiac parameters, each animal was injected with dye only once during the experiment (either during normoxia or during one of the hypoxic conditions). In order to compare flow values between $P_{O_2}$ treatments flow values for each vessel were normalized to $V_b$. ANOVA (Sigma Stat 2.0) was used to compare differences in flow to the AA, ALA and PA at $P_{O_2}$ values of 20.2 kPa, 13.7 kPa, 10.3 kPa and 6.8 kPa $O_2$. All values are reported as means ± SE with (n) indicating the number of animals.
Results

Cardiac parameters: cardiac output, stroke volume and heart rate

Under normoxic conditions $f_h$ and $V_b$ were significantly greater in gravid ($f_h = 266 \pm 7 \text{ bts min}^{-1}; V_b = 36 \pm 1.9 \text{ l/min}; n=19$) than non-gravid ($f_h = 241 \pm 7 \text{ bts min}^{-1}; V_b = 29.8 \pm 0.9 \text{ l/min}; n=11$) grass shrimp ($p \leq 0.05$), with no significant difference in $V_s$ (Fig. 2).

Values for $f_h$, $V_s$ and $V_b$ for gravid females exposed to hypoxia are displayed in figure 3. There were no significant differences in $f_h$, $V_s$ or $V_b$ for animals exposed to 13.7 kPa or 10.3 kPa $O_2$ as compared to their normoxic values, however there was a significant decrease in $f_h$ at 6.8 kPa $O_2$ ($f_h_{20.5} = 271 \pm 11; f_h_{6.8} = 229 \pm 16 \text{ beats min}^{-1}; p = 0.009$) that contributed to the significant decrease in $V_b$ ($V_b_{20.5} = 40.5 \pm 0.72, V_b_{6.8} = 29.9 \pm 0.2 \text{ l/bt}; p<0.001$). While stroke volume appeared to decrease at 6.8kPa, the drop was not significant.

Respirometry

Between 16-20 kPa $O_2$, average oxygen uptake for gravid grass shrimp was $21.8 \pm 1.35 \text{ mol/g-hr} (n=20)$. Values for critical oxygen tension ranged from 5.66 to 8.0 kPa $O_2$ with a mean value of 7.0 kPa $O_2 (n=17)$.

Comparison of the sum of arterial flow using dye injection with video-microscopic dimensional analysis for the determination of cardiac output

Student's t-test, revealed no significant differences between $V_b$ calculated from the sum of arterial flows using dye injection ($69.7 \pm 4.9 \text{ l/min}; n=6$) as compared to $V_b$ calculated from video-microscopy dimensional analysis ($74.1 \pm 3.3 \text{ l/min}; n=11$) ($t_{15} = 0.76; p=0.46$).
Hypoxia-induced changes in arterial hemolymph flow

Changes in arterial flow are summarized in Table 1. Although there were no significant differences in cardiac parameters except in animals exposed to 6.8 kPa O₂, there are significant changes in hemolymph flow at and below 13.7 kPa (Fig. 4). Flow decreased significantly to the anterior aorta as P O₂ was lowered from normoxic values down to 6.8kPa O₂ (F(3, 42) = 4.864; p=0.005). Tukey’s multiple comparisons revealed a significant drop in flow from values obtained at 20.5 kPa and 13.7kPa O₂ and those obtained at 10.3 and 6.8kPa O₂ (p<0.05). Flow to the anterior lateral arteries decreased as well (F(3,47) = 16.907; p<0.001) with a significant decrease in flow from values at 20.5 kPa O₂ to those obtained at 13.7 and 10.3 kPa O₂ (p<0.05) and another significant drop at 6.8 kPa O₂ (p<0.05). Flow to posterior aorta fell significantly (F(3,43) = 10.707 p<0.001) but not until 6.8 kPa O₂ (p<0.05).

The relative importance of oxygen/nutrient supply to an arterial system during hypoxic exposure was compared by expressing flow to each system as a percentage of total Vb (Table 1 and Fig. 5). The percentage of Vb delivered to the AA dropped significantly from 9.8% at 20.5 kPa O₂ to 7.7% at 10.3 kPa O₂ but then increased again to near normoxic values of 9.4% at 6.8 kPa O₂. There was a significant decrease in flow to the ALA’s from 22.0% at 20.5 kPa O₂ to 18.0% at 13.7 and 10.3 kPa O₂ with an additional drop to 15.7% at 6.8 kPa O₂. Flow to the PA remained at approximately 11% until P O₂ was dropped to 6.8 kPa when flow decreased significantly to 9.0%. Sternal artery flow values, calculated and expressed as a percentage of Vb, indicate a steady increase in flow to the SA...
during hypoxic exposure from 57.43% at 20.5 kPa O$_2$ to 65.89% at 6.8 kPa O$_2$.
The decreases in flow to the AA, ALA and PA contributed to the increase in flow to the SA.

Discussion

In this study we find that gravid female grass shrimp *P. pugio* have higher cardiac output values than their non-gravid counterparts, under normoxic conditions. We also found that oxygen tolerance appears to be reduced in gravid females exposed to hypoxia when compared with values reported in the literature. Finally, although there are no significant differences in cardiac output until 6.8 kPa O$_2$, we begin to see alterations in hemolymph flow at and below 13.7 kPa O$_2$.

Differences in cardiac parameters between gravid and non-gravid females

In crustaceans, oocyte maturation requires vitellogenesis: the biosynthesis of proteins and lipids and their transport and storage within the ovary (Quackenbush, 2001). Biosynthesis of these molecules is an extra energy cost associated with reproduction that would increase metabolic demand in gravid females. In comparing cardiac output values between gravid and non-gravid shrimp, we found a significantly higher cardiac output for gravid females that we attribute to the increased metabolic demand of their reproductive state.

Hypoxia-induced changes in cardiac parameters in gravid females

In contrast to the hypoxia-induced tachycardia reported by Harper and Reiber, (1999), in the present study, gravid grass shrimp demonstrate a hypoxia-
induced bradycardia, which is more typical of decapod crustaceans. In most cases, previous studies in grass shrimp and other crustaceans, do not account for reproductive state. The hypoxia-induced bradycardia reported in crabs, lobster and crayfish (Airriess and McMahon, 1994; Reiber and McMahon, 1998). There was a 20% decline in $f_h$ within 30 min of hypoxic exposure at 4.63 kPa O$_2$ in the crab *C. magister* (Airriess and McMahon, 1994), a significant decrease in $f_h$ occurred at 5.33 kPa O$_2$ in the crayfish, and a similar result in lobster, with a hypoxia-induced bradycardia below 10.0 kPa O$_2$ (Reiber and McMahon, 1998). In all cases, bradycardia was attenuated by an increase in $V_s$ that allowed $V_b$ to be maintained until reaching a $P_{crit}$ value. In the present study, the significant drop in $f_h$ at 6.8kPa, was not compensated for by the anticipated increase in $V_s$. This inability to increase $V_s$ may be the result of physical constraints on the cardiac sinus due to reproductive state. The paired ovaries are located in the cephalothorax and lie in a dorsal position above the cardiac stomach and hepatopancreas (McLaughlin 1983). They extend anterior ventrally beyond and below the pericardial sinus. The gravid females used in this study were stage 3 and 4 with the ripe ovaries extending into the space above the cardiac stomach. Ovaries ripe with eggs may impinge upon the pericardial sinus and limit expansion of the ventricle, producing a physical constraint on cardiac contraction due to limitation of space around the cardiac sinus.

**Respirometry**

In addition to the potential physical constraint on $V_s$, cardiac output and $P_{crit}$ may be limited due to the increased metabolic demand of vitellogenesis.
Based on our respirometry experiments, gravid females had higher $P_{\text{crit}}$ than values previously reported in the literature. However, previous reports did not differentiate between sex and/or life stage of the population used so a mixed population is assumed. Even so, our value for oxygen uptake (22 $\mu$mol/g·hr) is elevated compared to literature values. In respirometry experiments controlling for both high and low CO$_2$ concentrations, oxygen uptake was less than 20 $\mu$mol/g·hr between 17.5 to 20.5 kPa O$_2$ (Cochran and Burnett, 1996). Harper and Reiber (1999) used $V_b$ to estimate a $P_{\text{crit}}$ value of 5.3 kPa (~40 Torr) while Cochran and Burnett (1996), using respirometry, reported $P_{\text{crit}}$ values for of approximately 4.67 kPa for $P. pugio$. A higher rate of oxygen uptake and a higher $P_{\text{crit}}$ in our population, coupled with the significant differences in cardiac output between gravid and non-gravid females in this study, implies an increase in metabolic demand for gravid females.

Comparison of the sum of arterial flow using dye injection with video-microscopic dimensional analysis for the determination of cardiac output

The Fick principle, thermal dilution and dye dilution techniques have been used to estimate $V_b$ and volume flow in Crustacea (Belman, 1975; Blanchford, 1971; Burnett et al., 1981). More recently, the pulsed Doppler technique has proved to be a useful tool in studying flow patterns of brachyurans and macrurans as well as the redistribution of flow in response to stresses such as hypoxia, salinity changes, and neurohormones (Airriess, 1992; McGaw and Reiber, 1998; McGaw and McMahon, 1996, 1998). However, this technique is not amenable to very small animals with even smaller vessel diameters. Grass
shrimp, with their transparent exoskeleton, present a unique opportunity to investigate flow through the use of video-microscopy and dimensional analysis for the measurement of $f_h$ and $V_s$ (Harper and Reiber, 1999). Additionally, the location of the vessels just below the surface of the transparent carapace allows for measurement of hemolymph flow as well as measurement of internal vessel diameter in vivo.

Resting heart rate values were observed less than one hour after handling although two hours was allowed for acclimation during experimental procedures. Cardiac output calculated from the sum of arterial flows compared well with $V_b$ determined by dimensional analysis of the heart. The two values were not significantly different therefore calculations from dye fronts were used to calculate the distribution of flow in the grass shrimp.

**Hypoxia-induced changes in arterial flow**

Flow patterns through the arterial system, as measured using pulsed Doppler, vary between species, as does the response to hypoxia. Flow patterns have been studied extensively in brachyurans with limited information on the more primitive macruran body form which has a robust abdominal region. While both have five arterial systems leaving the heart, there are some notable differences in the proportion of hemolymph delivered to each system. The largest difference is in the percentage of $V_b$ distributed to the posterior aorta and the paired hepatic arteries. The hepatic arteries of brachyurans are of a larger diameter and receive 15-20% of $V_b$ in *C. magister* (McGaw and McMahon, 1998) and as much as 25-30% in *C. sapidus* (Reiber and McMahon, 1998). However,
Reiber and McMahon (1998) considered flow to the hepatic artery in the macrurans, *P. clarkii* and *H. americanus*, negligible due to its small diameter. Similarly, with less than 1% of V_b going to the hepatic arteries in *P. pugio*, flow from these vessels was not included in any calculations for V_b in the present study. The differences in flow patterns between the macrurans and brachyurans, justify a comparison only with other macrurans.

The anterior aorta supplies the cephalic region (brain) and eyestalks (sinus gland) in decapod crustaceans (Maynard, 1960; McMahon, et al., 1997). The reports of flow distribution to the anterior aorta are variable; one study reported anterior aortic flow values of 13% and 20% of V_b for *H. americanus* and *P. clarkii* respectively (Reiber et al., 1997), while Reiber and McMahon (1998) report values of 26% and 10.2% in the same species. This variability extends to results for hypoxic experiments as well. In the crayfish, as water P<sub>O2</sub> decreased, flow to the anterior aorta increased from 26% to as much as 33% of V_b until reaching 3.33 kPa O<sub>2</sub> at which point flow falls to about 10% of V_b (Reiber and McMahon, 1998). Gravid grass shrimp delivered nearly 10% of V_b to the anterior aorta during normoxia with a significant drop to 7.7% at 10.3 kPa O<sub>2</sub>, whereas at 6.8kPa O<sub>2</sub>, flow recovered to near normoxic levels (9.36%) (Table 1, Fig. 5).

Macrurans have a robust muscular abdominal region compared to brachyurans, so the posterior aorta receives as much as 20-25% and 10-12% of V_b in the lobster and crayfish respectively (Reiber et al., 1997; Reiber and McMahon, 1998). In the crayfish, hemolymph flow to the posterior aorta showed a steady decline with hypoxia, but the decrease was not significant until 3.33 kPa
O₂ (Reiber and McMahon, 1998). In the grass shrimp, flow to the posterior aorta decreased significantly at 6.8 kPa O₂ indicated by a drop from approximately 11% to 9% of total cardiac output. While this drop was statistically significant, an average of 10% of Vb continues to be delivered to this vessel. The animals may maintain flow to the abdominal region in an effort to escape hypoxic waters. Other Macrurans such as lobster and crayfish exhibit a robust tail flip escape response; this was not observed in grass shrimp during these experiments, however we observed occasional movement of the abdominal pleopods at all levels of hypoxia.

The ovaries receive hemolymph from the anterior lateral arteries (Maynard, 1960). Branches of the anterior lateral artery supply the supraesophageal ganglion and optic ganglia anteriorly (Maynard, 1960; Sandeman, 1967), but more importantly for this study, these vessels have branches to supply the hepatopancreas, and the thoracic viscera in general (Maynard, 1960; McMahon, et al, 1997). The site of vitellogenin synthesis, and hence an increase in metabolic demand, resides in the ovary in Procambarus, the hepatopancreas in Macrobrachium rosenbergii, and in both locations in Homarus americanus (Tsukimura, 2001). Even when synthesis occurs in the ovaries, lipids from the hepatopancreas are transported as lipoproteins within the hemolymph to the ovaries (Ravid, Tietz, Khayat, Boehm, Michelis and Lubzens, 1999). So, flow through the anterior lateral arteries may be increased to enhance oxygen delivery for the protein and lipid synthesis required during vitellogenesis.
and also to enhance the transport of molecules from the site of synthesis to the site of use and storage.

In the only Macruran in which flow to the anterior laterals has been measured (H. americanus) less than 4% of V_b was delivered during normoxia and this increased to 6% during moderate hypoxia (Reiber and McMahon, 1998). In the present study, 22% of V_b was delivered to the anterior lateral arteries in gravid grass shrimp in normoxic conditions. Although flow decreased significantly to this vessel during hypoxic exposure, it still received nearly 16% of total V_b even at 6.8 kPa O_2, much greater than recorded for H. americanus during normoxic conditions. While flow was redirected to the sternal, the animals still maintained a proportionately large amount of V_b to the anterior laterals, presumably to support the developing eggs.

The consequence of reduced flow from the arterial systems discussed thus far is that hemolymph flow is increased through the sternal artery. The sternal artery exits the heart ventrally and divides into the anterior thoracic artery and posterior abdominal artery that perfuse the ventral nerve cord. A portion of the anterior thoracic artery branches off further to perfuse the muscles of the scaphognathite used in ventilation (Bauman, 1921; Maynard, 1960). In both brachyurans and macrurans, the sternal artery receives the greatest percentage of V_b ranging from 45-65% (McGaw and McMahon, 1996, 1998; Reiber, et al, 1997; Reiber and McMahon, 1998). Hemolymph flow to the sternal artery remained unchanged in crayfish from 20.0 kPa O_2 down to 3.33 kPa O_2 at approximately 63% of cardiac output, however in the lobster there was an
increase from a normoxic value of 56.2% to 64.0% at 4.0 kPa O\textsubscript{2} (Reiber and McMahon, 1998). In gravid grass shrimp, flow to the sternal artery increased with hypoxia from 57.4% at 20.5 kPa to 65.9% at 6.8 kPa O\textsubscript{2}. These results are well within the range of the previously reported studies. A redirection of flow from other arterial systems, primarily the anterior aorta and anterior lateral arteries, enhances flow to the sternal artery.

The sternal artery via the anterior thoracic artery, supplies the musculature of the scaphognathite. During hypoxic exposure there is an increase in both the rate and depth of scaphognathite beating (McMahon and Wilkens, 1983; Airriess and McMahon, 1994) which results in an increase in ventilatory volume and therefore an increase in O\textsubscript{2} supply to the gas exchange surface. The additional energy requirement for these muscles coupled with increased flow through the gills necessitates an increase in hemolymph flow to the sternal artery.

**Summary**

When faced with hypoxic challenge, organisms must find a way to balance tissue perfusion with metabolic tissue demand. One way to accomplish this is to make compensatory changes in cardiovascular parameters. Our data suggest an increase in metabolic demand for gravid versus non-gravid females. We also found that gravid grass shrimp respond to hypoxia in much the same manner as other decapods crustaceans, with a hypoxia-induced bradycardia however, the increased metabolic demand of reproduction results may result in a failure to maintain $V_b$ and $P_{crit}$ at comparable levels to those reported in the literature.
(Harper and Reiber, 1999; Cochran and Burnett, 1996). Regardless of species or reproductive state, the current data in conjunction with previous studies suggests that decapod crustaceans, including the gravid grass shrimp, require enhanced oxygen delivery to the sternal artery. Survival depends on the ability to enhance oxygen uptake. Increased flow through the sternal artery enhances hemolymph and oxygen delivery to the respiratory apparatus and the gills and as well as the ventral nerve cord and other nervous tissue. While a proportionately large amount of flow is directed toward the anterior lateral arteries and hence the ovaries, ultimately, the ability of the female to survive the hypoxic stress demands an increase in flow to vital areas such as the respiratory apparatus and nervous tissue via the sternal artery.

Acknowledgements

This research is supported by NSF Grant 9874534 awarded to CLR and UNLV Graduate Student Association grant awarded to JAG.
References


Figure legends

Figure 1: Lateral view of shrimp showing the location of the five arterial systems, ovary, hepatopancreas and nervous tissue. (modified from McLaughlin, 1983)

Figure 2: Differences in cardiovascular parameters between non-gravid and gravid *P. pugio* at 20.5 kPa. (*) significant difference between the two groups, p<0.05.

Figure 3: Differences in $f_h$, $V_s$ and $V_b$ in gravid females exposed to three different levels of hypoxia. Solid bars always indicate normoxia, while shaded bars indicate the hypoxic condition noted on the x-axis. (*) significantly different from normoxia, p<0.001.

Figure 4: Changes in flow to the anterior aorta (AA), anterior lateral artery (ALA) and posterior aorta (PA) with decreases in oxygen tension. (a) sig. dif. from 20.5kPa, (b) sig. dif. from 6.8kPa; p < 0.05.

Figure 5: Changes in flow with decreases oxygen tension as a percentage of cardiac output: anterior aorta (AA), anterior lateral artery (ALA), posterior aorta (PA), sternal artery (SA)
Figure 1

Anterior Lateral Artery
Anterior Aorta
Supra-esophageal ganglion
Sinus gland
Hepatic Artery
Hepatopancreas
Ventral Nerve Cord
Sternal Artery
Anterior Ventral Artery
Posterior Ventral Artery
Posterior Aorta
Heart
Figure 2

Heart Rate

Stroke volume

Cardiac Output

Non-gravid  Gravid

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Figure 4

- Anterior Aorta
- Anterior Lateral
- Posterior Aorta

Flow (μl/min) vs. PO$_2$ (kPa)

PO$_2$ (kPa): 6.8, 10.3, 13.7, 20.5

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Table 1: Change in flow due to hypoxic exposure as determined from video analysis of dye front movement. Values are normalized to cardiac output for each treatment (mean ± SE in Dl/min). ANOVA used to determine significance between the treatment groups (a sig. dif. from 20.5kPa; b sig. dif. from 6.8kP; p < 0.05). Values for the sternal artery are calculated values (see text). Numbers in parentheses are percentage of cardiac output being delivered to the arterial system.

<table>
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<tr>
<th>PO2 (%)</th>
<th>Vb (% Vb)</th>
<th>AA</th>
<th>AL</th>
<th>POST</th>
<th>STERNAL</th>
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<tr>
<td>20.5kPa</td>
<td>39.62</td>
<td>3.9 ± 0.34</td>
<td>8.7 ± 0.43</td>
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<td>13.7kPa</td>
<td>35.67</td>
<td>3.2 ± 0.28</td>
<td>6.7 ± 0.52&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.0 ± 0.26&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>10.3kPa</td>
<td>34.97</td>
<td>2.7 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.0 ± 0.36&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.9 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>6.8kPa</td>
<td>29.9</td>
<td>2.8 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.7 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
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CHAPTER 4

ASSESSMENT OF THE PRESSURE-AREA RELATIONSHIP OF THE SINGLE VENTRICLE OF THE GRASS SHRIMP, PALEAMONETES PUGIO

This chapter has been prepared for publication in the Journal of Comparative Physiology and is presented in the style of that journal. The completed citation is:

Abstract

The ventricular pressure-area (P-A) relationship has been used extensively to study the mechanics and energetics of multi-chambered hearts of closed circulatory system vertebrates. In the current study we apply the use of P-A 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 these ventricles include multiple ostia entering and multiple arterial valves 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 P-A loops. Based on the P-A loops generated in this study, the ventricle of *P. pugio* processes the same primary phases of the cardiac cycle as ventricles from the multi-chambered hearts of vertebrates: (1) iso-volmic contraction (2) ventricular emptying (3) iso-volmic relaxation and (4) ventricular filling. The area enclosed by the P-A loop provides a measure of stroke work and when multiplied by heart rate, cardiac work. This initial examination of P-A loops from a single ventricle decapod crustacean demonstrates the utility of this technique to further elucidate the cardiac mechanics and energetics of this system in particular during times of physiological stress.
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. A pressure-area (P-A) loop provides a mechanism for the assessment of myocardial stroke work and hence myocardial O₂ consumption. The P-A loop relationship has been used extensively to study the energetics of cardiac contraction in the ventricles of mammalian and avian systems, yet this tool has not yet been applied to the study of cardiac dynamics in the single ventricles of animals possessing open circulatory systems.

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 co-ordinate the opening and closing of multiple ostial and aortic valves. Hemolymph enters the heart through three pairs of muscular ostia (Fig. 1 B, C), and leaves the heart via six aortic valves that lead to five
arterial systems (Fig 1A). Pre-branchial hemolymph from active tissues 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).

Physiologically, the initiation of cardiac contraction in the single ventricle of a decapod is neurogenic, as compared to the myogenic properties of vertebrate cardiac myocytes, and is driven by a burst of action potentials from the cardiac ganglion located in the inside dorsal surface of the heart (Florey, 1960; Sullivan and Miller, 1984). Overall cardiac function depends on ganglionic burst frequency and duration (see Cooke, 2002 for review), which is further altered by both cardio-excitatory and cardio-inhibitory nerves originating from the central nervous system. Despite 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\textsuperscript{2+} necessary for contraction. As in all muscle cells, relaxation occurs when released Ca\textsuperscript{2+} is sequestered back into the SR or pumped out to the extra-cellular 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). Given these similarities, myocardial O\textsubscript{2} consumption, or cardiac work, would be similar for both vertebrate myocytes and decapod myocytes at the tissue level.

The P-A loop (Fig. 2) provides a tool for the estimation of myocardial O\textsubscript{2} consumption. There are four distinct phases that include (1) iso-volmic contraction as pressure is generated by the ventricle, (2) ventricular emptying as the pressure in the ventricle overcomes peripheral pressure, (3) iso-volmic 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. While ventricular area and pressure can be determined independently for the determination of stoke volume, or total pressure change, the integration of this data is the basis for determining myocardial O\textsubscript{2} consumption. Once integrated, the area enclosed by a P-A 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 P-A loops from the single
ventricle of a decapod crustacean. These loops would allow for a detailed assessment of stroke work in a single ventricle that has multiple inflow and outflow valves. We used the grass shrimp, *P. pugio* to test the hypothesis that P-A loops from this multi-outlet single ventricle, would be comparable to the P-A loops generated from the ventricle of the vertebrate closed circulatory system.

**Materials and Methods**

*Animal preparation:* Grass shrimp, *Palaemonetes pugio*, were purchased from GulfSpecimen Marine Laboratories, Inc., and maintained in 20L aquaria in aerated seawater (30-32ppt 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). 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 & Reiber, 1999). The transparent exoskeleton allows for the measurements of area and pressure in vivo.

**Experimental design**

Sea water (30 ± 2 ppt) within a flow through experimental chamber was maintained at 20°C and water P<sub>O2</sub> 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 (P0₂=20.5 kPa) and acclimated for one hour. Thereafter a minimum of three recordings of pressure and volume were made for each animal.

**Intra-ventricular pressure**

Intra-ventricular pressure was measured using a servo-null pressure system (model 900A, World Precision Instruments, Sarasota, FL) and an analog-digital board (DAQPad 6020-50E, National Instruments, Austin, TX) at a rate of 600Hz. A glass micro-pipette with a 2-5 μm diameter tip was filled with 3M NaCl and positioned in the ventricle with the use of a micromanipulator (World Precision Instruments). The micro-pipette 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 3M NaCl-filled pipette tip and prevents changes in resistance by generating an opposing pressure to the pressure present at the tip. Intra-ventricular pressure was calculated as the difference between the measured pressure within the ventricle and the zero-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 60Hz using a stereo-microscope (Leica MZ12.5, McBain Instruments) equipped with a video camera (World Precision Instruments), frame grabber...
board (LG-3, Scion, Frederick, MD) and programmed frame grabbing software (Scion Image, Scion, Frederick, MD). 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 were 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 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 sec by an output trigger to the AD board and the frame capturing board. Using a custom computer program (K. Tobita using LabView, National Instruments, Inc.) the pressure waveform was decimated from 600Hz to 60Hz and interpolated with the image data to yield a series of x, y co-ordinates required for the P-A loop.
Statistical analysis

Values are means ± SE (n = 12). Heart rate ($f_h$), maximum pressure ($P_{max}$), minimum pressure ($P_{min}$), change in pressure ($\Delta P$), maximum area ($A_{max}$), minimum area ($A_{min}$) and change in area ($\Delta A$) were determined by analyzing the pressure and video output from LabView (Nat'l Instruments) using a customized computer program MATLAB (The Mathworks, Inc., Natwick, MA). After interpolation of the pressure-area data to generate multiple P-A loops in LabView, the data was analyzed using MATLAB to obtain an average P-A loop as well as the area enclosed by the average P-A loop. The area of the P-A loop is an estimate of stroke work. Minute stroke work is the product of stroke work per beat and heart rate.

Results

Independent measures of pressure and area allow for assessment of the cardiac cycle, prior to the data points being integrated to form a P-A loop. Two representative cardiac cycles are shown in figure 4. Time between pressure peaks was used to determine $f_h$. Mean heart rate for 6 cardiac cycles (n=12 animals) is $285 \pm 29$ bts min$^{-1}$ with $53 \pm 0.75\%$ of time spent in systole and $47 \pm 0.75\%$ of time spent in diastole. The average change in pressure was $29.4 \pm 1.2$ mmHg with mean $P_{max}$ and $P_{min}$ values at $20.9 \pm 1.3$ and $-8.5 \pm 0.7$ mmHg, respectively. The heart of the grass shrimp is modeled as a trapezoid (Harper and Reiber, 1999) allowing for the conversion of area volume. Mean $V_s$ was
102.2 ± 3.2 μl bt⁻¹ and 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 8 P-A loops (Fig. 5C). The x-y data was then imported into MATLAB to yield a single, averaged loop. The MATLAB program calculates the area enclosed by the P-A loop (Fig 5D) which is used as an index of stroke work (SW) (mean = 0.748 ± .014 mmHg*mm²). The P-A loop does not account for time, therefore, the product of area and \( f_h \) yields an estimate of cardiac work (CW). When the pressure and area data are integrated, the P-A loop generated from the grass shrimp ventricle has the same four phases seen in Fig. 2 and will be discussed in detail.

**Discussion**

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

While all four phases are present in the P-A 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 bt 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.

End-diastolic volume (area) (EDA) is affected by venous filling pressure, distensability of the ventricular wall, and time available for filling (Sagawa, et al., 1988). When comparing ventricular EDA in an open circulatory system to that of a ventricular EDA 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. The open circulatory system does not have a direct venous return path; nor as in multi-chambered hearts is there atrial contraction to enhance filling of the ventricle. 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, 1993). 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 (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 iso-volmic contraction phase therefore requires overcoming peripheral resistance along with the nervous coordination of the timing and tension in the individual valves.

The emptying phase of the cycle is biphasic in all P-A 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 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. While we do not have specific data on the sequential opening of the arterial valves, the biphasic nature of the pressure tracings may be due to 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 iso-volmic phase with all valves closed and a rapid drop in pressure toward zero. This iso-volmic 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 crustacea, 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, 1994). The microscopic architecture and excitation-contraction mechanism of decapod crustaceans is similar to cardiac myocytes of mammals (Shinozaki et al, 2002; Yazawa et al, 1999). 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 is converted to suction during the succeeding systole. Titin has been described in striated muscles of invertebrate species including crayfish (Fukuzawa et al,2002). This molecule, like mammalian titin, gains its functional elasticity from the random coil region (Fukuzawa et al., 2002).

In addition to the potential role of titin, the heart of decapod crustaceans may have an additional external mechanism to generate such 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 due to 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 would appear to have available both active (recoil) and passive (∆P) properties during the filling phase.

The area enclosed by the PA 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. Figure 7 is an example of a PA loop from an animal under normoxic conditions and how the PA loop changes after 20 minutes of hypoxic exposure (6.2kPa O₂). During hypoxic exposure the area enclosed by the P-A loop decreases (Guadagnoli, Ch. 5). Based on the hypoxic P-A loop, the pressure difference is decreased, stroke volume is increased and, overall, total P-A loop area 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 due to 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 P-A loops in evaluating the cardiac
response to stress 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 P-A loops. Given the ongoing study of physiological stressors and interactions in this model, the use of P-A loops provides a new tool for researchers to evaluate multiple levels of ventricular function in the open-circulatory system of decapod crustaceans.

Acknowledgements

The authors would like to thank Jason Vance for his expertise in writing the necessary programs in MATLAB for the evaluation of the pressure and area data and its integration into P-A loops.
References


Volk EL (1988) The role of suspensory ligaments in modifying cardiac output in crustaceans. MSc Thesis, University of Calgary, Alberta


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Figure legends

Figure 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; HA – hepatic artery; PA – posterior aorta; SA – sternal artery; VOV – ventral ostial valve; DOV – dorsal ostial valve; LOV – lateral ostial valve).

Figure 2: Pressure-volume loop of the left ventricle for a single cardiac cycle. (Adapted from Berne and Levy, 1986)

Figure 3: (A) outline of heart in systole defines the minimal area (B) outline of heart in diastole defines maximal area. The area between the maximal and minimal area defines the region of interest used in automated area analysis.

Figure 4: Pressure and area tracing for two cardiac cycles. EDA – end-diastolic area; ESA – end-systolic area.

Figure 5: (A) Pressure tracing, (B) changes in area with each cardiac cycle calculated from ROI (C) eight P-A loops generated by combining the values from A and B. (D) The result of MATLAB averaging of the eight loops and calculation of P-A loop area.
Figure 6: Representative P-A loop from after MATLAB analysis. ESA – end-systolic area; EDA – end-diastolic area; the grey shaded area is a period of ventricular "sucking."

Figure 7: An example of P-A loops from one animal under normoxic conditions (20.5kPa) and 20 minutes of hypoxia (6.2kPa).
Intra-cardiac pressure
Exceeds peripheral pressure

Ventricular emptying

Iso-volumetric contraction

End-diastolic volume

Iso-volumetric relaxation

Stroke work

End systolic volume

Ventricular volume (ml)

All valves closed

50 ml 120 ml

Ventricular volume

50 ml 120 ml

Ventricular pressure (mmHg)

0 50 100

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Figure 4

Emptying phase

Filling phase

Isovolumic contraction

Isovolumic relaxation

Pressure (mmHg)

Area (mm²)

Time (sec)

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Figure 5

A

B

C

D

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Figure 6
Figure 7

Ventricular area (mm²)

Intra-ventricular pressure (mmHg)

-15 -10 -5 0 5 10 15 20 25

-15 -10 -5 0 5 10 15

0.74 0.76 0.78 0.80 0.82 0.84 0.86 0.88

20.5 kPa

6.8 kPa (20min)
CHAPTER 5

CHANGES IN CARDIAC PERFORMANCE DURING HYPOXIA IN THE GRASS SHRIMP, *PALEAMONETES PUGIO*

This chapter has been prepared for publication in the American Journal of Physiology and is presented in the style of that journal. The completed citation is:


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Abstract

The amount of work performed by the ventricle is a function of heart rate and contractility. Crustaceans experience a hypoxia induced bradycardia that is thought to result in an overall reduction of cardiac work; however, this hypothesis has not yet been tested and is the primary purpose of this study. Pressure and area data were obtained simultaneously and in vivo under normoxic (20.2 kPa O₂) and hypoxic (6.8 or 2.2 kPa O₂) 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 heart rate, an estimate of cardiac work and myocardial O₂ consumption. Changes in dp/dt are correlated to the isovolmic contraction phase and also provide an indication of stroke work. At both levels of hypoxia, intra-cardiac pressure, dp/dt, stroke work and cardiac work fell significantly. Significant decreases in intra-cardiac pressure provide the primary mechanism for the decrease in stroke work and the hypoxia induced bradycardia contributes to the overall fall in cardiac work.

Compared to normoxic P-A loops, at both levels of hypoxia, P-A loops become curvilinear indicating a fall in peripheral resistance. Overall, hypoxia induced bradycardia coupled with a reduction in intra-cardiac pressure serves to reduce stroke work and cardiac work, reducing myocardial O₂ consumption during times of oxygen lack.

Keywords: ventricular function, decapod crustacean, diastolic suction, suspensory ligaments
Introduction

The work performed by a 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, and is reflected in stroke work. As the ventricle pumps to match oxygen supply and demand, either heart rate or stroke volume may be altered in an effort to maintain cardiac output at an appropriate level. Hypoxic stress in most crustaceans results in a hypoxia induced bradycardia that is thought to result in an overall reduction of cardiac work; however, this hypothesis has not yet been tested in crustaceans, the main purpose of this study.

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, ventilatory rate and changes in $O_2$ binding properties of respiratory pigments (McMahon, 2001; Reiber and McMahon, 1998; Wheatly and Taylor, 1981). Typically, crayfish, crabs and lobsters, exhibit a hypoxia-induced bradycardia with a concomitant increase in stroke volume (McMahon, 2001; Reiber et al, 1992; Reiber, 1995; Reiber and McMahon, 1998; Wheatly and Taylor, 1981). The increase in stroke volume may result from an increased filling time and allows the animal to maintain cardiac output near normoxic values in the face the hypoxia. Intracardiac, infra-branchial and pericardial sinus pressures have been shown to increase in the crayfish upon exposure to hypoxia. While many studies have examined alterations in independent factors...
upon hypoxic exposure, the data thus far 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 \( f_{hi} \), \( V_s \), cardiac output (\( V_b \)) and intra-cardiac pressure, we integrate the independent measures of ventricular area and intra-cardiac pressure to generate pressure-area loops (P-A loops) to determine changes in cardiac energetics during the hypoxic response.

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 co-ordinate the opening and closing of multiple ostial and aortic valves. Hemolymph enters the heart through three pairs of muscular ostia (Fig. 1 B, C), and leaves the heart via six aortic valves that lead to five arterial systems (Fig 1). Pre-branchial hemolymph from active tissues 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).

Beyond contractile force of the ventricle, alterations in peripheral resistance within the arterial system can play a role in the required amount of stroke work. 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). The redistribution of arterial hemolymph flow is accomplished by muscular cardio-arterial valves located at the entrance to each arterial system (Maynard, 1960). The ability to redistribute hemolymph flow through these valves has been demonstrated in several species (Bathynomus Kihara and Kuwasawa, 1984; Cancer magister McGaw et al., 1994; Panulirus japonicus Kuramoto and Ebara, 1984a; Procambarus clarkii Reiber, 1994). Beyond the cardio-arterial valves the vasculature does not contain smooth muscle (Shadwick et al, 1990) to allow for changes in peripheral vascular resistance. 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 (Wilkens, Davidson and Cavey, 1997; Wilkens and Taylor, 2003). The exoskeleton of decapod crustaceans may also impose a limitation to changes in peripheral resistance, however, in crayfish, shrimp and lobster, abdominal flexion
and extension would provide an additional mechanisms for altering the internal resistance of the animal (Reiber et al, 1997, Taylor, 1990), and therefore stroke work.

Physiologically, the initiation of cardiac contraction in the single ventricle of a decapod is neurogenic, as compared to the myogenic properties of vertebrate cardiac myocytes, and is driven by a burst of action potentials from the cardiac ganglion located in the inside dorsal surface of the heart (Florey, 1960; Sullivan and Miller, 1984). Overall cardiac function depends on ganglionic burst frequency and duration (see Cooke, 2002 for review), which is further altered by both cardio-excitatory and cario-inhibitory nerves originating from the central nervous system. Despite 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 to 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 at al, 2002) with activation of voltage-dependent sarcolemmal Ca$^{2+}$ release channels that allow the entry of Ca$^{2+}$ necessary for contraction. As in all muscle cells, relaxation occurs when released Ca$^{2+}$ is sequestered back into the SR or pumped out to the extra-cellular 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). Given these
similarities, myocardial O$_2$ consumption, or cardiac work, would be similar for both vertebrate myocytes and decapod myocytes at the tissue level.

In the current study 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 ($f_h$), stroke volume ($V_s$) and cardiac output ($V_b$). By integrating the pressure and area data, we generated P-A loops to evaluate changes in cardiac work in the ventricle of the decapod crustacean, the grass shrimp, $P$. $pugio$, during hypoxic exposure.

Materials and Methods

Animal preparation

Grass shrimp, $Palaemonetes pugio$, were purchased from GulfSpecimen Marine Laboratories, Inc., and maintained in 20L aquaria in aerated seawater (30-32ppt 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 (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). The video camera was
placed over the chamber so that digital images of the heart could be captured through the transparent exoskeleton (see methods from Harper & Reiber, 1999).

**Experimental Design**

Sea water (30 ± 2 ppt) 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). All animals were initially placed in the experimental chamber in normoxic water (P\textsubscript{O\textsubscript{2}} = 20.5 kPa) and acclimated for one hour. A minimum of three recordings of pressure and volume were made for each animal during normoxic conditions.

Hypoxic conditions were achieved by controlled mixing of nitrogen and air using a gas mixing system (Cameron Instruments). Critical oxygen tension for *P. pugio* is 4.7 (Harper and Reiber 1999) to 5.3 kPa (Cochran and Burnett 1996), we therefore choose hypoxic conditions that would bracket this P\textsubscript{crit} value. Water P\textsubscript{O\textsubscript{2}} in the flow through chamber was lowered to either 6.8 kPa O\textsubscript{2} or 2.2 kPa O\textsubscript{2}.

Simultaneous measurements of pressure and area where made 5, 10, 15, 20 and 30 min after exposure to hypoxia.

**Intra-ventricular pressure**

Intra-ventricular pressure was measured using a servo-null pressure system (model 900A, World Precision Instruments, Sarasota, FL) and an analog-digital board (DAQPad 6020-50E, National Instruments, Austin, TX) at a rate of 600Hz. A glass micro-pipette with a 2-5 μm diameter tip was filled with 3M NaCl and positioned in the ventricle with the use of a micromanipulator (World Precision Instruments). The micro-pipette 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 3M NaCl-filled pipette tip and prevents changes in resistance by generating an opposing pressure to the pressure present at the tip. Intra-ventricular pressure was calculated as the difference between the measured pressure within the ventricle and the zero-pressure recorded when the tip was placed in the experimental chamber at a level adjacent to the heart (Tobita & Keller, 2000).

**Video image processing**

Video images were acquired in vivo through the transport exoskeleton at a rate of 60Hz using a stereo-microscope (Leica MZ12.5, McBain Instruments) equipped with a video camera (World Precision Instruments), frame grabber board (LG-3, Scion, Frederick, MD) and programmed image acquisition software (Scion Image, Scion, Frederick, MD). Each video frame was analyzed to determine ventricular area 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 (Fig. 2 A & 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 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 area within the ROI and the minimum ventricular area.

The pressure signal (600 Hz) (Fig. 2D) and video images (60 Hz) (Fig. 2E) were acquired simultaneously for 4 sec by an output trigger to the AD board and the image acquisition board. Using a custom computer program (K. Tobita using LabView, National Instruments, Inc.) the pressure waveform was decimated from 600Hz to 60Hz 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 minimums ($A_{min}$) is equivalent to end-systolic area (EDA). Plotting both the pressure and area data on a single x-y coordinate system yields 8 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) after 5, 10, 20 and 30 min (Sigma Stat 9.0). If significant, 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 ($\Delta P$), first derivative of maximal pressure ($dp/dt$), maximum area ($A_{max}$), minimum area ($A_{min}$) and change in area ($\Delta A$) were determined by analyzing the pressure and video output from LabView (Nat'l Instruments) using a customized computer program (J. Vance) in MATLAB (The Mathworks, Inc., Natwick, MA). All values are means ± SE.

For the determination of stroke volume, $A_{max}$ and $A_{min}$ were converted to volume. The heart was modeled as a trapezoid \[ d \frac{1}{2} (h + a) \] 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 0.64$h$ during systole and 0.67$h$ 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 pressure-area data to generate multiple P-A loops in LabView, the data was analyzed using MATLAB to obtain the average P-A loop of 5-12 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 kPa O₂ and 2.2 kPa O₂ respectively. In grass shrimp exposed to 6.8 kPa O₂, $f_H$ falls significantly after 5 min of hypoxic exposure ($F_{(4,59)} = 8.52; p<0.001$) and remains unchanged for the remainder of the hypoxic trial (Fig. 4A). Changes in $V_s$ are not statistically significant, yet the slight increase above normoxic values may be biologically relevant in helping maintain $V_b$ (Fig. 4A) in the face of declining $f_H$. When grass shrimp are exposed to 2.2 kPa O₂ (Fig 4B), $f_H$ falls significantly after 5 minutes ($F_{(4,49)} = 25.7; p<0.001$). Changes in $V_s$ are not significant and the significant drop in $V_b$ ($F_{(4,49)} = 27.5; p<0.001$) is reflected by changes in $f_H$ (Fig 4b). After 20 min of exposure to hypoxia there is a second drop in both $f_H$ and $V_b$. Recovery values (35 min) were obtained for several animals after a return to normoxia (6.8 kPa $n = 4$; 2.2 kPa $n = 6$). While these values do not appear to be different from normoxic values at time 0 min, due to the small number of samples these data were not included in statistical analysis (Fig 4A and 4B).

Pressure changes are much more dramatic than changes in $f_H$, $V_s$, or $V_b$ at 6.8 kPa O₂. The fall in $P_{\text{max}}$ ($F_{(4,59)} = 17.0; p<0.001$), and increase in $P_{\text{min}}$ ($F_{(4,59)} = 7.8; p<0.001$) results in a decrease in intra-cardiac $\Delta P$ ($F_{(4,59)} = 23.2; p<0.001$) after only 5 minutes of hypoxic exposure with a second drop in $\Delta P$ after 10
minutes of hypoxic exposure (Fig 5A). Interestingly, at 2.2 kPa O\(_2\) the changes in \(\Delta P\) (\(F_{(4,49)} = 7.7; p<0.001\)) after 5 minutes result from changes in \(P_{\text{max}}\) 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 and 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 at 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 Figs 7A and 8A. At 6.8 kPa O\(_2\) mean P-A loop area does not drop significantly, however cardiac work (CW), the product of \(f_{\text{H}}\) 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 \(f_{\text{H}}\). At 2.2 kPa O\(_2\) (Fig 8B) CW drops significantly (\(F_{(4,49)} = 33.8; p<0.001\)) after 5 min of hypoxic exposure due to the previously mentioned drop in \(f_{\text{H}}\) coupled with the significant fall in P-A loop area (\(F_{(4,49)} = 13.6; p<0.001\)).

Discussion

Within the single ventricle of the grass shrimp, \textit{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) iso-
volmic contraction (2) ventricular emptying (3) iso-volmic relaxation and (4) ventricular filling and is therefore 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 \( f_h, V_s, V_b \) and pressure

As with many decapod crustaceans, grass shrimp exhibit hypoxia induced bradycardia. In these animals, we did not observe an increase in \( V_s \) as expected, despite the maintenance of \( V_b \) 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 hours of hypoxia at a level of 6.8 kPa O\(_2\) (Guadagnoli and Reiber, 2005) and in this study, \( V_b \) was maintained near normoxic values with increases (though not statistically significant) in stroke volume. At 2.2 kPa O\(_2\), a value well below the animal’s \( P_{\text{crit}} \) value (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 was 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. While 2.2 kPa O\(_2\) is below the grass shrimp’s \( P_{\text{crit}} \) value, 30 min of hypoxic stress is not sufficient to hinder the grass shrimp’s ability to recover.

Intra-cardiac pressure has been measured previously in decapod crustaceans weighing from 12g (Gnathophausia ingens) to over 500 grams.
(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 \( f_r \) from 90-180 bts min\(^{-1}\) (Belman, 1975). In this study we measure intra-cardiac pressure in vivo in a milligram sized decapod crustacean and provide the first evidence of negative pressures during diastole. Maximal diastolic intra-cardiac pressure and \( \Delta P \) dropped rapidly upon exposure to both levels of hypoxia. Intra-cardiac, infra-branchial and pericardial sinus pressures were shown to increase upon exposure to hypoxia in crayfish (Reiber and McMahon, 1998). However, a fall in intra-cardiac pressure alone during hypoxic exposure, would increase the passive \( \Delta P \) for filling of the ventricle. While we did not measure pericardial sinus pressures, Wilkens and McMahon (1992) have found that changes in pericardial sinus pressure do not change end-diastolic volume. Overall, any increase in the passive \( \Delta P \) may serve to reduce SW.

Maximal dp/dt\(_{\text{Max}}\) is a descriptor of iso-volmic pressure and a fall dp/dt\(_{\text{Max}}\) is associated with a fall in myocardial O\(_2\) consumption (Saguwa et al., 1988). A fall in dp/dt\(_{\text{Max}}\) was observed during both levels of hypoxic exposure with a more dramatic fall during severe hypoxia. Since the ventricle is a highly aerobic organ, any conservation of myocardial O\(_2\) consumption would be beneficial when an animal is exposed to a low O\(_2\) environment. The reduction in myocardial O\(_2\) consumption is supported by the decrease in SW as determined from P-A loops. Examination of the P-A loops under both hypoxic conditions, reveals that the decrease in SW results primarily from the dramatic fall in \( \Delta P \).
The cardiac cycle and P-A loops

During the cardiac cycle the ventral pericardial membrane is contracted and hence depressed during diastole and relaxes during systole creating 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 iso-volmic contraction phase ostial valves and arterial valves are closed. In decapod crustaceans, flow to the seven arteries leaving the ventricle that is regulated by six muscular bicuspid valves. The valves prevent passive reflux of hemolymph during diastole, but actively control outflow during systole via neural innervation (Alexandrowicz, 1932; Kuromoto and Ebara, 1984). Both excitatory and inhibitory neurons are present in the valves (Kuromoto et al., 1992) with neural excitation causing valve muscle contraction that impedes flow and neural inhibition causing relaxation that facilitates flow (Wilkens, 1997). The ventricle must not only generate sufficient pressure to overcome resistance in the vasculature to open the valves (afterload), but the force required can also be altered depending on the contractile state of the valves. The iso-volumic contraction phase therefore requires overcoming peripheral resistance along with the nervous coordination of the timing and tension in the individual valves.
During the relaxation phase, pressure approaches zero and becomes negative and there is a period of “diastolic sucking” as the ventricle begins to fill during negative pressure (Kraner, 1959) and completes its filling at low, but positive pressures. Negative pressures have not yet been reported in crustacea, 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, 1994). The microscopic architecture and excitation-contraction mechanism of decapod crustaceans is similar to cardiac myocytes of mammals. 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 is converted to suction during the succeeding systole. Titin has been described in striated muscles of invertebrate species including crayfish (Fukuzawa et al., 2002). The primary difference in the molecules is that the mammalian form spans the entire sarcomere form Z-disc to M-line while the crayfish molecule spans the A-zone. This molecule, like mammalian titin, gains its functional elasticity from the random coil region (Fukuzawa et al., 2002).

In addition to the intrinsic effects of titin, the heart of decapod crustaceans may have an additional external mechanism to generate such a restoring force. The decapod crustacean heart is held within the pericardial sinus via suspensory ligaments that stretch during systole and recoil during diastole. 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 would appear to have available both active (recoil) and passive (ΔP) properties during the filling phase.

The area enclosed by the P-A loop is an indicator of SW. Analysis of SW is useful in determining the efficiency of the cardiac contraction and how this may change under various conditions (Sagawa et al., 1988). The significant fall in SW at 2.2 kPa O$_2$ appears to be largely due to the large drop in pressure, since 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 included, CW (min$^{-1}$), drops significantly. These data support a decrease in myocardial O$_2$ consumption during hypoxic exposure.

Two plausible explanations for the reduction in myocardial O$_2$ consumption will be addressed here: a fall in peripheral resistance and/or a change in suspensory ligament tension. During hypoxia scaphagnathite frequency increases enhancing water flow through the branchial chambers (Wheatly and Taylor, 1981). Decapod crustaceans are enclosed in an exoskeleton and do not possess musculature in the majority of their vessels, thus limiting the ability to alter internal total peripheral resistance. During hypoxia an increase in forward ventilation via scaphagnathite beating, serves to enhance gill perfusion potentially reducing internal peripheral resistance (Taylor 1990). Another option for decreasing internal resistance is the extension or retraction of the tail region (Taylor 1990; Reiber 1997). 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 P-A loops generated during the hypoxic trials become progressively curvilinear, 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 due 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). Oxygen demand of these small muscles would be substantially less then oxygen consumption of the ventricle and may provide an additional mechanism for conservation of O$_2$ consumption.

Although changes in $V_a$ were not significant, the slight increases may be biologically relevant in allowing the animal to maintain $V_b$. The accompanying
decrease in $f_H$ allows for increased filling time and reduces CW. The combination of these factors serves to reduce myocardial O\textsubscript{2} utilization during exposure to 6.8 kPa O\textsubscript{2} while still maintaining $V_b$. The drop in myocardial O\textsubscript{2} consumption at 2.2 kPa O\textsubscript{2} may result from the same factors noted previously. Hypoxic stress at 2.2 kPa O\textsubscript{2} is well below the $P_{crit}$ value for grass shrimp so the animal may be forced to reduce overall metabolism as evidenced by the significant drop in $V_b$.

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 in 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.

Acknowledgements

The authors would like to thank Jason Vance for his for writing the programs necessary to evaluate the pressure and area data and its integration into P-A loops.
References


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Volk EL (1988) The role of suspensory ligaments in modifying cardiac output in crustaceans. MSc Thesis, University of Calgary, Alberta


Figure legends

Figure 1: (A) Overview of circulatory anatomy of a shrimp. (B) Dorsal view of heart (C) Lateral of view of heart. (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).

Figure 2: (A) outline of heart in systole defines the minimal area (B) outline of heart in diastole defines maximal area. (C)The area between the maximal and minimal area defines the region of interest (ROI) used in automated area analysis. (D) Pressure tracing, (E) changes in area with each cardiac cycle calculated from ROI (F) eight P-A loops generated by combining the values from d and e.

Figure 3: Representative P-A loop obtained from averaging the eight P-A loops from figure 2f using MATLAB. ESA – end-systolic area; EDA – end-diastolic area; the grey shaded area is a period of ventricular “sucking.”

Figure 4: Cardiac parameters obtained from shrimp exposed to (A) 6.8 kPa (n=12) and (B) 2.2 kPa (n=10). (a) SD from time 0 at 20.5kPa; (b) SD from 10 min. (p<0.001).
Figure 5: Pressures obtained from shrimp exposed to (A) 6.8 kPa (n=12) and (B) 2.2 kPa (n=10). (a) SD from time 0 at 20.5kPa; (b) SD from 10 min. (p<0.001).

Figure 6: Changes in ventricular dp/dt at 6.8 and 2.2 kPa O\textsubscript{2}. (a) values at 6.8 kPa that are SD from normoxic values. (b) values at 2.2 kPa that are SD from normoxic values.

Figure 7: (A) Representative P-A loops from a single animal under normoxic conditions (20.5kPa) and after 20 and 30 min of hypoxia at 6.8 kPa. PA loop areas are 0.5682, 0.5092, 0.5168 for normoxia, 20 and 30 min at 6.8 kPa respectively. (B) Relationship between $f_h$, P-A loop area and minute CW. (a) SD from time 0 at 20.5 kPa (p<0.001).

Figure 8: (A) Representative P-A loops from a single animal under normoxic conditions (20.5kPa) and after 10, 20 and 30 min of hypoxia at 2.2 kPa. P-A loop areas are 1.175, 0.867, 0.854 and 0.848 for normoxia, 10, 20 and 30 min at 2.2 kPa respectively. (B) Relationship between $f_h$, P-A loop area and minute CW. (a) SD from time 0 at 20.5 kPa (b) SD from 5 min at 2.2 kPa (c) SD from 10min at 2.2 kPa (p<0.001).
Figure 2

(a) Area Systole
(b) Area Diastole
(c) ROI
(d) Pressure
(e) Area
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Figure 5

a

Pressure (mmHg)

\( P_{\text{Max}} \)
\( P_{\text{Min}} \)
\( \Delta P \)

Time (min) at 6.8 kPa

b

Pressure (mmHg)

\( P_{\text{Max}} \)
\( P_{\text{Min}} \)
\( \Delta P \)

Time (min) at 2.2 kPa

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Figure 6

![Graph showing dp/dt (mmHg/min) over time at hypoxia (min) for two conditions: 6.8 kPa and 2.2 kPa. The graph includes error bars and annotations denoted by letters a and b.]

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Figure 8

(a) Ventricular area (mm$^2$) @ 2.2 kPa

(b) $f_H$ (bts min$^{-1}$), work (area$\times f_H$), PA loop area (mm$^2$*mmHg)

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Table 1: Changes in cardiovascular parameters upon exposure to 6.8 kPa. Data expressed as mean standard error of the mean. Statistical significance assigned based on p<0.05. (n = 12; 204.2 ± 9.2 mg)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normoxia 20.2 kPa</th>
<th>5 min</th>
<th>10min</th>
<th>20min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bts min⁻¹)</td>
<td>285 ± 13</td>
<td>263 ± 13*</td>
<td>260 ± 12*</td>
<td>260 ± 12*</td>
<td>253.0 ± 11*</td>
</tr>
<tr>
<td>End-diastolic volume (μl)</td>
<td>572.8 ± 14.5</td>
<td>595.2 ± 17.8</td>
<td>573.1 ± 18.8</td>
<td>566.9 ± 19.4</td>
<td>563.3 ± 22.3</td>
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<tr>
<td>End-systolic volume (μl)</td>
<td>470.6 ± 12.8</td>
<td>487.7 ± 15.2</td>
<td>466.4 ± 18.0</td>
<td>463.7 ± 18.9</td>
<td>459.4 ± 21.3</td>
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<tr>
<td>Stroke volume (μl bt⁻¹)</td>
<td>102.2 ± 3.2</td>
<td>107.4 ± 3.7</td>
<td>106.7 ± 2.3</td>
<td>103.2 ± 2</td>
<td>103.9 ± 1.9</td>
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<tr>
<td>Cardiac output (ml min⁻¹)</td>
<td>29.04 ± 1.5</td>
<td>28.0 ± 1.3</td>
<td>27.7 ± 1.5</td>
<td>26.9 ± 1.4</td>
<td>26.08 ± 4.9</td>
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<tr>
<td>$P_{\text{max}}$ (mmHg)</td>
<td>20.9 ± 1.3</td>
<td>18.1 ± 1.4*</td>
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<td>16.2 ± 1.1*</td>
<td>16.1 ± 1.1*</td>
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<tr>
<td>$P_{\text{min}}$ (mmHg)</td>
<td>-7.5 ± 0.7</td>
<td>-6.4 ± 0.3*</td>
<td>-6.2 ± 0.3*</td>
<td>-5.9 ± 0.5*</td>
<td>-6.0 ± 0.7*</td>
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<td>ΔP (mmHg)</td>
<td>29.4 ± 1.8</td>
<td>24.3 ± 1.6*</td>
<td>22.6 ± 1.4*</td>
<td>22.1 ± 1.3*</td>
<td>22.2 ± 1.1*</td>
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<tr>
<td>$dp/dt_{\text{Max}}$ SW (mm²•mmHg)</td>
<td>3.8 ± 0.6</td>
<td>2.7 ± 0.4*</td>
<td>2.2 ± 0.4*</td>
<td>2.3 ± 0.4*</td>
<td>2.3 ± 0.5*</td>
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<td>CW (mm² • mmHg min⁻¹)</td>
<td>0.75 ± 0.14</td>
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<td>213.0 ± 29.4</td>
<td>197.1</td>
<td>171.8</td>
<td>153.1</td>
<td>153.3 ± 21.4*</td>
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Table 2: Changes in cardiovascular parameters upon exposure to 2.2 kPa. Data expressed as mean standard error of the mean. Statistical significance assigned based on p<0.05. (n = 10; 213.4 ± 16.6 mg)

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<th>Parameter</th>
<th>Normoxia 20.2 kPa</th>
<th>5 min</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bts min⁻¹)</td>
<td>277 ± 3</td>
<td>244 ± 9*</td>
<td>224 ± 8*</td>
<td>203 ± 7*</td>
<td>203.4 ± 7.4*</td>
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<tr>
<td>End-diastolic volume (µl)</td>
<td>644.8 ± 38.3</td>
<td>657.8 ± 31.5</td>
<td>667.6 ± 33.0</td>
<td>662.3 ± 33.4</td>
<td>646.7 ± 31.3</td>
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<tr>
<td>End-systolic volume (µl)</td>
<td>510.3 ± 30.8</td>
<td>520.6 ± 26.7</td>
<td>530.4 ± 28.4</td>
<td>524.8 ± 27.0</td>
<td>522.2 ± 27.9</td>
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<tr>
<td>Stroke volume (µl b⁻¹)</td>
<td>134.5 ± 8.2</td>
<td>139.2 ± 7.8</td>
<td>137.2 ± 6.4</td>
<td>137.4 ± 8.4</td>
<td>124.6 ± 5.7</td>
</tr>
<tr>
<td>Cardiac output (ml min⁻¹)</td>
<td>37.2 ± 1.52.3</td>
<td>34.6 ± 2.3*</td>
<td>31.2 ± 41.8*</td>
<td>28.4 ± 2.2*</td>
<td>25.4 ± 1.7</td>
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<tr>
<td>Peak systolic pressure (mmHg)</td>
<td>24.0 ± 2.5</td>
<td>18.7 ± 2.0*</td>
<td>18.4 ± 2.6*</td>
<td>17.7 ± 2.7*</td>
<td>17.0 ± 2.5*</td>
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<td>Minimal diastolic pressure (mmHg)</td>
<td>-6.2 ± 0.6</td>
<td>-5.3 ± 1.1</td>
<td>-6.0 ± 1.2</td>
<td>-5.9 ± 1.3</td>
<td>-6.30 ± 1.4</td>
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<tr>
<td>Change in pressure (mmHg)</td>
<td>31.1 ± 3.1</td>
<td>23.0 ± 1.8*</td>
<td>24.4 ± 2.2*</td>
<td>23.5 ± 2.3*</td>
<td>23.5 ± 1.8*</td>
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<tr>
<td>dp/dt Max Stoke work</td>
<td>3.8 ± 0.5</td>
<td>2.0 ± 1.6*</td>
<td>1.8 ± 0.2*</td>
<td>1.5 ± 0.2*</td>
<td>1.6 ± 0.3*</td>
</tr>
<tr>
<td>P-A loop area (mm²•mmHg)</td>
<td>1.21 ± 0.15</td>
<td>1.01 ± 0.13</td>
<td>0.87 ± 0.09*</td>
<td>0.80 ± 0.09*</td>
<td>0.68 ± 0.07*</td>
</tr>
<tr>
<td>Minute cardiac work (mm²•mmHg min⁻¹)</td>
<td>332.6 ± 37.8</td>
<td>241.1 ± 27*</td>
<td>193.9 ± 21*</td>
<td>165.4 ± 2.2*</td>
<td>140.8 ± 17.9*</td>
</tr>
</tbody>
</table>
CHAPTER 6

ENVIRONMENTAL HYPOXIA INFLUENCES HEMOGLOBIN SUBUNIT COMPOSITION IN THE BRANCHIOPOD CRUSTACEAN, TRIOPS LONGICAUDATUS

This chapter has been published in the Journal of Experimental Biology and is presented in the style of that journal. The completed citation is:


DOI: 10.1242/jeb.01794

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Summary

Hemoglobin (Hb) is a highly conserved protein that provides a vital link between environmental oxygen and its use and/or storage within an organism. While ubiquitous among vertebrates, Hb occurs frequently in invertebrate phyla as well. Many Arthropods species use the copper binding pigment hemocyanin, but unique in this phylum are the branchiopod crustaceans which express Hb. Branchiopod Hb concentration and structure are exquisitely sensitive to environmental oxygen availability. Hemoglobin concentration and oxygen binding affinity increase with decreasing oxygen tension in *Daphnia*, *Artemia* and *Triops*. The change in binding affinity is attributed to differential Hb subunit expression in *Daphnia* and *Artemia*, but remains unclear for *Triops*. This is the first study to demonstrate developmental plasticity of Hb subunit expression in a notostracan, *Triops longicaudatus*, reared under conditions of varying oxygen availability. In response to variable oxygen environments, *T. longicaudatus* differentially express four primary Hb subunits ranging between 30-34 kDa, with normoxic-reared animals expressing primarily the heavier subunits and hypoxic-reared animals expressing increased proportions of the lower molecular weight subunits. Moreover, differential Hb subunit expression is induced upon transfer of normoxic-reared adults to a hypoxic environment, such that the distribution of Hb subunits in the transferred adults becomes similar to that of hypoxic-reared animals. Two-dimensional gel electrophoresis and follow-up analyses revealed several iso-forms of Hb subunits that may represent differential gene expression and/or post-translational modification. Unlike *Daphnia* and *Artemia*, the Hb
hypoxic response is not reversible in that there is no significant decrease in Hb concentration or change in Hb subunit expression pattern when hypoxic-reared adults were transferred to a normoxic environment.
Introduction

Hemoglobin (Hb) is a highly conserved protein that provides a vital link between environmental oxygen and its use and/or storage within an organism (Weber and Vinogradov, 2001). A ubiquitous oxygen transport molecule among vertebrates, Hb occurs frequently in invertebrate phyla as well. Among the Arthropods, many animals use the copper binding pigment hemocyanin, but unique in this phylum are the brachiopod crustaceans which express Hb. Within this group, the concentration and structure of Hb is exquisitely sensitive to environmental oxygen availability, especially in the Anastraca (Artemia) and Cladocera (Daphnids) (Weber, 1980; Peters et al., 1990; Terwilliger, 1998). However, little is known about the developmental and hypoxia-dependent kinetics of Hb expression in Notostraca, the subject of this study.

Hemoglobin structure varies widely among branchiopods. It is a large extra-cellular molecule ranging from 220 kDa in Artemia salina (Moens and Kondo, 1978) to nearly 800 kDa in Lepidurus apus lubbocki (Ilan and Daniel, 1979) and is composed of various sized subunits. Among the Cladocera and Notostraca, Hb subunit chains range from 30-37 kDa (Peeters et al., 1990), with two heme groups per chain (Ilan and Daniel, 1979). In the Notostracans Lepidurus apus lubbocki and Lepidurus bilobatus, the native Hb molecules have molecular weights of approximately 798 kDa and 680 kDa, respectively, with subunits in the 33 – 34 kDa range (Dangott and Terwilliger, 1979; Ilan and Daniel, 1979). Triops longicaudatus Hb has a molecular weight of ~600 kDa, and
Horne and Beyenbach (1974) estimated the molecular weight of the subunits at approximately 20.5 kDa.

The natural history of many of crustaceans includes regular bouts of hypoxia, the response to which includes, but is not limited to, increased ventilation and perfusion over oxygen exchange tissues, increased cardiac output and/or reduction in metabolism and the demand for oxygen (Wheatly and Taylor, 1981; Hochachka and Lutz, 2001). During periods of chronic hypoxia certain branchiopods such as *Daphnia magna* (Fox, 1955; Zeis, 2003) *A. salina* (Gilchrist, 1954; Heip et al, 1978) and *T. longicaudatus* (Scholnick and Snyder, 1996; Harper, 2003) increase Hb content; this response has been observed in both experimental and natural populations (Kobayashi and Hoshi, 1982; deWachter et al., 1992). Moreover, branchiopods modify Hb structure and functional properties in response to hypoxia (Wolf et al., 1983; Zeis et al., 2003). In *D. magna* and *T. longicaudatus*, hypoxia induces an increased Hb oxygen-binding affinity (Wolf et al., 1983; Kobayashi et al., 1994; Harper, 2003; Zeis et al., 2003). The branchiopod hypoxic response may also include differential Hb subunit assembly (Kimura et al., 1999) and differential subunit expression, as demonstrated in *D. magna* (Zeis et al., 2003).

In this study we assess the hypoxic response and plasticity of Hb expression in a Notostracan by examining differences in Hb concentration and subunit expression in *T. longicaudatus* reared under hypoxic or normoxic conditions. We show that Hb subunit expression changes significantly during developmental hypoxia and when normoxic-reared adults are transferred to a
hypoxic environment. Interestingly, the adult response is apparently fixed, as a reversal to normoxic Hb expression patterns does not occur when hypoxic-reared adults are returned to a normoxic environment.

Materials and Methods

Animal rearing

Tadpole shrimp cysts were collected from an ephemeral pool in Brownstone Canyon, Clark County, Nevada. Cysts were dried for at least 30 days at 40°C and re-hydrated in 10-liter aquaria filled with either normoxic or hypoxic water. Normoxic conditions were maintained by bubbling room air into the aquaria (20 kPa O₂). A gas mixing flow meter (GF-3, Cameron Instruments, Port Aransas, TX) which provided a mixture of 90% nitrogen and 10% air, was used to maintain hypoxic conditions at 2-5 kPa O₂. Water temperature was maintained at 25 ± 1°C using submersible aquarium heaters. Newly hatched larvae were fed ground spirulina for the first two days and then were switched to finely ground goldfish pellets. All animals were exposed to a 14:10 light/dark cycle.

Hemolymph collection, purification and protein content

Hemolymph samples were collected from sexually mature females (12-13 days after re-hydration). To induce a hypoxic response in adults, females were reared to sexual maturity under normoxic conditions and on day 12 were transferred to hypoxic conditions (5 kPa O₂). A normoxic hemolymph sample was collected on day 12 (prior to transfer) and hypoxic hemolymph samples were
collected once every 24 hrs post-transfer for seven days. To assess Hb changes in hypoxic-reared adults subsequently exposed to normoxia, the reverse experiments were conducted in which female *Triops* reared to sexually maturity under hypoxic conditions were switched to normoxic conditions.

Hemolymph samples were collected from adult animals (12-20 days old) via dorsal puncture of the heart. To do so, animals were netted, blotted dry and the carapace folded back to reveal the dorsal location of the heart. The heart was punctured with a 28-guage needle and hemolymph samples were collected into capillary tubes, transferred into ice-cold micro-centrifuge tubes and frozen at −20 °C until use. As previously demonstrated by Horne and Beyenbach (1971), the major detectable protein in *Triops* hemolymph is hemoglobin. To confirm this samples were partially purified on a 10 cm gel exclusion column packed with Sephacryl S-300 (Amersham Biosciences, Piscataway, NJ, USA) in buffer containing 0.05 M Tris, 0.1 M NaCl, 0.01 M MgCl2 and 1mM PMSF and collected in 0.5ml fractions. Absorbance peaks of each fraction were measured at 280 nm for the determination of protein and 415 nm for determination of hemoglobin. Hb samples from both normoxic and hypoxic reared animals were run through the column and these partially purified samples were run on SDS-PAGE gels and compared to non-purified samples. After confirming that the primary protein in *Triops* hemolymph is hemoglobin, protein concentrations of whole hemolymph samples were used to estimate changes in hemolymph hemoglobin content. Protein content was determined colorimetrically based on the method of Bradford with a kit from BioRad, (500-0001, Hercules, CA, USA).
One dimensional gel electrophoresis

SDS-PAGE was performed using the Protean II xi Gel Electrophoresis system (BioRad) with a 10% separating and 4% stacking gel as described by Laemmli (1970). After determination of protein content, hemolymph was diluted in sample buffer containing 62.5 mM Tris HCl (ph 6.8), 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% β-mercaptoethanol. Samples were heated at 95 °C for 5 min. and then loaded onto gels. One lane of each gel was loaded with Precision Plus Dual Color Protein Standards (Bio-Rad #161-0374) with a molecular weight range from 250 kDa to 10 kDa. Specifically, molecular weight markers at 25 kDa and 37 kDa were used for determination of the molecular weight of the Hb bands. Following electrophoresis, proteins were visualized using Coomassie Brilliant Blue and scanned on a Typhoon 9410 Phosphorimager (Amersham Biosciences).

Two-dimensional gel electrophoresis

Hemolymph samples containing 200 µg protein were added to 300 µl of re-hydration media consisting of 7.9M urea, 4.0% CHAPS (w/v), 84.4mM DTT, 35mM Tris base, 0.0025% bromophenol blue, 2M thiourea, and 0.8% 3.5-10 ampholytes, and shaken for 2 hours at 30°C. Isoelectric focusing was performed using pre-cast gel strips with a 3-10 immobilized pH gradient (Amersham Biosciences). The samples were then applied to the gel strips and the strips were re-hydrated overnight. Proteins were focused (Multiphor IEF, Amersham Biosciences) at 500V for 30 min, 1500V for 1hr, 2500V for 1hr and 3500V for 48hrs. Gel strips were removed and laid perpendicularly over a 10% SDS-PAGE
to separate the proteins by molecular weight. Second dimension gels were stained with Coomassie Brilliant Blue and scanned on a Typhoon 9410 Phosphorimager (Amersham Biosciences) for later analysis of the spots using ImageQuant software (Amersham Biosciences).

**Two-Dimensional fluorescence difference gel electrophoresis (2D DIGE)**

The experimental design and protocol was followed as per manufacturer’s instructions (Amersham Biosciences). Briefly, CyDyes were reconstituted in 1.5 volumes of high grade N,N-dimethylformamide-d$_7$ (DMF) to a concentration of 400 pmol ml$^{-1}$ CyDye. One hemolymph sample each from a normoxic-reared and a hypoxic-reared animal was labeled with a fluorescence dye at a ratio of 50 μg protein labeled with 400 pmol fluor. Samples were labeled as follows: Cy2: a mixture hemolymph from a normoxic-reared and a hypoxic-reared adult; Cy3: the normoxic-reared hemolymph sample; Cy5: the hypoxic-reared hemolymph sample. The Cy2 labeled pooled sample served as an internal control on the gel for the DeCyder analysis.

After labeling, 50 μg of each of the three labeled samples was added to sample buffer (as described above in 2D gel methodology) and loaded onto pre-cast gel strips with a 3-10 immobilized pH gradient. The samples were then subject to 2D dimensional electrophoresis as described previously and scanned with the phosphorimager in the 2D DIGE mode at wavelengths appropriate for each of the CyDyes. All spot picking and image analysis of the gel was performed using DeCyder software (Amersham Biosciences) developed specifically for 2D-DIGE gel analysis using an internal standard experimental
design. DeCyder software scans the entire gel and outlines all detectable areas. Areas that are up-regulated greater than 2.5 times are outlined in blue and those down-regulated greater than 2.5 times are outlined in red.

**NH₂-terminal Sequencing**

Hemolymph proteins were isolated using 2D gel electrophoresis under denaturing conditions. Gels were electro-transferred to polyvinylidene fluoride (PVDF) membranes (TransBlot electrophoretic transfer cell, BioRad). Transferred spots were visualized with Coomassie Brilliant Blue, cut and sent to the Nevada Proteomics Facility. Sequence analysis was performed using automated Edman degradation on an Applied Biosystems (Foster City, CA) Precise 492 sequencer.

**Statistical Analysis**

A Student's t-test was used to determine the differences in Hb content between rearing groups and one-way ANOVA (Sigma Stat 3.2) was used to determine differences in Hb content in the transfer experiments. ANOVA was used to determine significant differences in subunit expression between normoxic and hypoxic-reared animals and the difference in the time course of subunit induction for the transfer experiments. All data passed the normality test and were equally distributed. Post hoc pairwise comparisons were made using Tukey's test. The level of significance was set at p<0.05 for all statistical analysis. Values are reported as means ± SE with (n) indicating the number of samples.
Results

Hemolymph purification and protein content

Absorbance of hemolymph fractions at 280nm (absorbance value for protein) coincided with the absorbance peaks at 415nm (absorbance value for Hb) suggesting that Hb is the predominant protein present in hemolymph samples (Fig. 1). We further confirmed this result by comparing one-dimensional SDS-PAGE gels of partially purified and non-purified hemolymph (data not shown). Since the primary bands on both gels were those of hemoglobin, samples were not purified prior to 1D- SDS-PAGE, 2D SDS-PAGE or 2D- DIGE.

Hypoxic-reared animals had a significantly greater [hemolymph protein] (37.7 ± 1.3 mg/ml; n=23) than normoxic-reared animals (29.3 ± 1.6 mg/ml; n=20; p<0.001) (Fig. 2). In the transfer experiments, normoxic-reared adults showed no change in [hemolymph protein] during the first 3 days after transfer to hypoxia, but significantly increased [hemolymph protein] 4 -7 days after transfer (F\textsubscript{2.25} = 5.14, p=0.01; Fig. 3A). In the reverse experiments, there were no significant differences in [hemolymph protein] when hypoxic-reared animals were transferred to normoxic conditions even after 14 days (Fig. 3B; F\textsubscript{2.23} = 0.99; p=0.4).

One-dimensional gel electrophoresis

One-dimensional gel electrophoresis revealed that Hb from normoxic-reared \textit{Triops} consisted of three primary subunits, two dark staining bands at 34kDa (Hba) and 33kDa (Hb\(\beta\)) and a lighter staining band at 32kDa (Hb\(\delta\)) (Fig. 4A, lanes 3 and 6). From ImageQuant analysis, the relative proportions of Hba,
Hbβ, and Hbδ in normoxic animals were 45.5 ± 1.3%, 37.3 ± 2.1% and 14.5 ± 1.1% of total Hb, respectively (n=7). A faint band at 30kDa (Hbγ), that accounts for less than 3% of total Hb subunit composition, was present in some normoxic reared animals. The same four bands are present in hypoxic-reared animals, but the relative contribution from each band changes dramatically (Fig. 4A, lane 12). The higher molecular weight subunits, Hbα and Hbβ drop to 30.9 ± 2.1% and 12.6 ± 1.6% of total Hb, respectively, whereas lower molecular weight subunits, Hbδ and Hbγ, increase their contributions to 33.4 ± 3.0% and 23.1 ± 1.9%, respectively (n=8).

When sexually-mature, normoxic-reared females were transferred to hypoxic water, induction of Hbγ was detected as early as 2 days post-transfer (Fig. 4A, lanes 4 and 5). With continued hypoxic exposure, the Hb composition of normoxic-reared adult females changes to match that of hypoxic-reared females, with a decrease in Hbα and Hbβ and an increase in Hbδ and Hbγ (Fig. 4A, lanes 7, 8, 10 and 11).

ImageQuant analysis of separated Hb subunits from four to six animals per treatment per day was used to determine the time course of Hb subunit induction in normoxic-reared animals that were transferred to hypoxia upon sexual maturity. The intensity of each band (Hbα, Hbβ, Hbδ and Hbγ) is expressed as a percentage of the total intensity of all the bands in each lane. The relative contribution of Hbα decreases significantly from normoxic controls by two days post-transfer, from 45.5 ± 1.3% to 34.8 ± 1.3% (F_{8,36} = 13.17, p<0.001), and thereafter is not different from hypoxic control values (Fig. 5A).
After three days of hypoxic exposure the contribution of Hbβ decreases significantly from the normoxic value of 37.3 ± 2.1% to 14.1 ± 1.9% ($F_{8,36} = 37.59$, $p<0.001$) and thereafter is not different from hypoxic-reared animals (Fig. 5B). Hbδ increases significantly by 3 days post-transfer, from 14.5 ± 1.1% to 25.3 ± 2.1% ($F_{8,36} = 12.27$, $p<0.001$) (Fig. 5C). The average value of Hbδ from 3 days post-transfer to 7 days post-transfer is 30.8 ± 1.4% a value not significantly different from hypoxic controls. The induction of Hbγ occurs by 3 days post-transfer, when it increases significantly from normoxic values of 2.78% to 25.1 ± 1.3% ($F_{8,36} = 23.01$, $p<0.001$) which is not significantly different from hypoxic-reared animals (Fig. 5D). These data collectively show that the Hb subunit composition of normoxic-reared, sexually-mature animals becomes indistinguishable from hypoxic-reared animals by 3 days after transfer to chronic hypoxia (Fig. 4A).

Similar plasticity in Hb subunit expression was not observed in hypoxic-reared Triops. Hypoxic-reared animals transferred to normoxia did not alter Hb subunit composition after 6 days; therefore the experiment was repeated and extended to 14 days. Even after 14 days of normoxic exposure, hypoxic-reared animals retained Hbγ and did not exhibit the Hb subunit pattern of normoxic reared animals (Fig. 4B).

**Two-dimensional gel electrophoresis**

Hemolymph samples collected from normoxic-reared, hypoxic-reared and hypoxic transferred Triops were run on 2D gels to further elucidate changes in Hb subunit expression. Representative gels of individual animals are shown in
Fig. 6. Based on an average of four 2D gels from each group, Hbδ and Hbγ increase from 12% and 0% in normoxic-reared animals to 28% and 22% in hypoxic-reared animals respectively. Each 2D gel revealed several iso-electric forms of Hb subunits suggesting a typical phosphorylation train (Halligan et al., 2004).

Two-Dimensional difference gel electrophoresis (2D DIGE) and image analysis

The use of 2D DIGE allows samples from different treatments to be run on the same gel, eliminating the problem of gel to gel variation, and allows comparisons to be made using DeCyder software (Amersham Biosciences) designed specifically for the analysis of 2D DIGE. An individual gel scan is shown in Fig. 7A. When the normoxic and hypoxic wavelength scans are combined, hypoxic animals clearly show differential expression of lower molecular weight subunits. The yellow spots (Fig. 7A top panel) are expressed in both normoxic and hypoxic-reared animals, while the green spots (those induced by hypoxia) are of lower molecular weight. These are the same lower molecular weight subunits that are significantly increased in both the 1D and 2D gels shown in Figs. 4 and 6. DeCyder analysis quantitatively confirms the up-regulation of lower molecular weight subunits. The spots outlined in blue (Fig. 7B upper panel) are up regulated 2.5 times or greater. The lower panel of Fig. 7B shows an example of DeCyder analysis of the spots. The spot outlined in pink shows a dramatic increase in volume in the hypoxic gel versus the normoxic gel.
NH₂-terminal sequencing

Spots were removed from 2D gels of normoxic and hypoxic reared animals for NH₂-terminal sequencing as shown in Fig. 6. Examination of NH₂-terminal amino acid sequences (Table 1) revealed differences in amino acid sequences between Hb subunits, with the sequences for Hbα, Hbβ and Hbd being distinctly different. The trains of spots that are of similar molecular weight have similar NH₂-terminal sequences supporting the hypothesis that these “trains” of spots are derived from post-translational events, perhaps phosphorylation. Both spots of Hbd have identical sequences, while there is minor Hbα spot sequence variation.

Discussion

This study clearly demonstrates that both developing and adult T. longicaudatus respond to hypoxia by altering Hb concentration and subunit composition. The correlation between lower oxygen tension and increased Hb concentration has been documented in many animal phyla including the branchiopods Daphnia (Green, 1956; Kobayashi & Hoshi, 1982; Kobayashi & Tanaka, 1991; Zeis et al., 2003) and Artemia (Gilchrist, 1955; DeWachter et al., 1992) and thus is not surprising in Triops. Our values for total hemolymph protein are greater than the [Hb] values previously reported in the literature for field captured Triops: 16.2 mg/ml by Horne and Beyenbach, (1971) and 7 mg/ml by Scholnick and Snyder (1996). Interestingly, when Triops were reared in laboratory conditions hemoglobin values were elevated compared to field values.
to 14 mg/ml in normoxic reared animals and 20-24 mg/ml in hypoxic reared animals (Scholnick and Snyder, 1996). Since the primary hemolymph protein is hemoglobin (Horne and Beyenbach, 1971), total hemolymph protein concentration was used to estimate relative changes in hemoglobin content. This would necessarily elevate the values reported in this study since previous reports determine hemolymph Hb levels based on the spectral properties of heme in hemolymph samples (Horne and Beyenbach, 1971; Scholnick and Snyder, 1996).

Increasing Hb concentration affords the animal an increase in oxygen carrying capacity (Gilchrist, 1955; Pirow et al., 2001) and in both A. salina and D. magna, the increase in Hb concentration during hypoxic stress is accompanied by an increased oxygen affinity (Heip, 1978; Kobayashi et al., 1988). Similarly, using whole hemolymph samples, under physiological conditions, T. longicaudatus hemoglobin demonstrates an increase in \( O_2 \) binding affinity from \( P_{50} = 1.14 \text{ kPa} \ O_2 \) in normoxic reared animals to \( P_{50} = 0.5 \text{ kPa} \ O_2 \) in hypoxic reared animals (1-3 kPa) (Harper, 2003). Horne and Beyenbach (1971) reported a \( P_{50} \) value of \( \sim 0.91 \text{ kPa} \ O_2 \) for hemolymph samples collected from field populations of T. longicaudatus.

The differential Hb subunit expression between normoxic and hypoxic-reared individuals may account for the differences in Hb oxygen binding affinity in hypoxic-reared individuals. When exposed to chronic hypoxia, normoxic-reared adult Triops alter their Hb subunit structure to match that of hypoxic-reared animals. During this transition, hypoxia induces a nearly 10 fold increase in Hb\( \gamma \)
by 3 days post-transfer and an average 3-fold increase in Hbδ. Variation in the concentration of these two subunits largely explains the increases in Hb concentration during hypoxia (Figs 5 and 7). DeCyder analysis indicates no down-regulation of the heavier subunits Hbα and Hbβ, and supports the findings of the ImageQuant analysis that the relative drop in Hbα and Hbβ is due to the up-regulation of the lower molecular weight subunits, Hbδ and Hbγ.

The relative contribution of Hbα drops 2 days post-transfer, but then stabilizes and continues to be an important component of Hb contributing over 30% to total Hb. Alternatively, the contribution of Hbβ drops sharply from 35% to an average of only a 15% contribution of total Hb. These changes, combined with those of Hbδ and Hbγ result in Hb profiles that do not differ between normoxic animals 3 days post transfer to a hypoxic environment and hypoxic-reared animals. Hypoxic induction of differential Hb subunit expression has been demonstrated in the Cladocera, *D. magna*, with both up and down-regulation of different Hb subunits (Kobayashi et al, 1988; Zeis et al., 2003). In *A. salina*, all three Hb subunits are up-regulated when adult animals are exposed to hypoxia, with the greatest increase in the subunit with the greatest O₂ affinity, Hb III (van den Branden et al., 1978), which is not normally present in adult animals (Heip et al., 1978; Vandenberg et al., 2002).

Separation of Hb on two dimensional gels revealed a number of isoelectric forms for three of the four different molecular weight subunits. The observed pattern was characteristic of post-translational phosphorylation (Halligan et al., 2004) and similar to a train of spots that has been reported for
Moina macrocopa and D. magna (Kimura et al., 1999; Kato et al., 2001; Zeis et al., 2003). While post-translation modification may play a role in regulating Hb oxygen affinity, these spots may just as likely be due to differential gene expression as already demonstrated in D. magna exposed to hypoxia (Kimura et al., 1999).

Sequencing of the NH$_2$-terminal was performed in an effort to assess whether the iso-electric forms were due to post-translational modification or differential gene expression. The differences in NH$_2$-terminal sequences between Hb$\alpha$, Hb$\beta$ and HB$\delta$ suggest that these Hb subunits are produced from different genes that may be differentially expressed upon hypoxic exposure. The similarity in sequence between Hb$\delta$ spots 1 and 3 suggests that these two spots have different iso-electric points due to post-translational modifications. The slight amino acid differences between Hb$\alpha$ 2, 4 and 5 are inconclusive and may be due to either post-translational modification and/or additional gene regulation. NH$_2$-terminal sequences of Hb in the Daphnid species, M. macrocopa, indicate at least three different subunits represented by three genes (Kato et al., 2001). This was confirmed by comparing the amino acid sequences with the amino acid sequences derived from the translation of nucleotide sequences of M. macrocopa Hb genes (Kato, et al., 2001). There are currently no genetic data available for such a comparison in Triops.

While differential Hb subunit expression is plastic during development and inducible in adulthood, the response was not reversed upon transfer of hypoxic-reared Triops to normoxia. Hypoxic-reared Triops, when returned as adults to a
normoxic environment, showed no changes in the pattern of Hb subunit expression up to 14 days after return to normoxia, nor did Hb concentration decrease significantly (Figs. 3B and 4B). In *D. magna* and *A. salina*, differences in Hb concentration are often associated with changes in coloration (Gilchrist, 1954; Kobayashi and Gonoï, 1985). Hypoxic reared adult *Triops* have a visibly deeper red coloration of their ventral appendages compared to their normoxic-reared counterparts. When normoxic-reared animals are transferred to hypoxia there is a visible increase in the redness of their ventral appendages; however, there is no obvious decrease in redness when hypoxic-reared animals are transferred to normoxia. In our experiments, coincident with the hatching of *Triops* we observed hatching of the anastrocan, *Thamnocephalus platyurus* and therefore transferred hypoxic-reared *T. platyurus* along with *Triops*. The *T. platyurus* response is bi-directional in that hypoxic-reared individuals show a decrease in color and reduction in hemolymph protein concentration from 28.3 ± 3.6 % to 8.4 ± 1.1 % after 10 days in normoxia; hypoxic-reared *T. platyurus* not transferred to normoxic conditions remain dark red (J. A. Guadagnoli, unpublished). Similarly, *A. salina* and *D. magna* had lower Hb concentrations after a return to high oxygen concentration (Kobayashi and Hoshi, 1982). The mechanism of Hb turnover and/or degradation in Branchiopods is not well understood, although hypoxic-reared *Triops* are clearly less responsive to a return to normoxia than *T. platyurus*, *A. salina* or *D. magna*.

Even so, *Triops longicaudatus* demonstrates remarkable developmental plasticity when reared in different oxygen environments, and a hallmark of this
response is differential Hb subunit expression. Adults transferred to hypoxia are sensitive to changes in oxygen tension which induce a change in Hb subunit composition that is similar to the Hb subunits expressed by hypoxic-reared animals. These subunits may be crucial to increasing Hb oxygen binding affinity of hypoxic animals. The recent discovery that hypoxia induced Hb synthesis in Daphnia magna is HIF (hypoxia inducible factor) dependent (Gorr et al., 2004) could explain the mechanism by which hypoxia induces differential subunit expression in Triops as well. The finding that Hb concentration and subunit expression are not reversed upon a return to normoxia in Triops merits further investigation into the possible mechanisms and regulation of Hb turnover in Branchiopods.

Acknowledgments

The authors would like to thank Dr. Frank van Breukelen for his knowledge and assistance in 2D gel electrophoresis and protein transfer, Ms. Kate Shen for her technical assistance in the laboratory techniques used in these experiments and Mr. Dan Harkness at Amersham Biosciences for his assistance in the DyCyder analysis. Edman sequencing was performed by the Nevada Proteomics Facility, part of the Nevada Biomedical Research Infrastructure Network (BRIN) which is supported by an award from the National Center for Research Resources (NCRR) (grant # 5P20RR016464-03). This research was also supported by NSF grant EPS-0132556 to SPR AND CLR and NSF EPS Graduate Research Grant to JAG.
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Figure Legends

Figure 1: Spectral analysis of *Triops* hemolymph fractions. The fraction with the greatest protein absorbance peak (280 nm) coincides with the fraction having the greatest Hb absorbance peak (415 nm).

Figure 2: Differences in hemolymph protein concentration between normoxic and hypoxic-reared adult females. Hypoxic-reared females have significantly more Hb than normoxic-reared individuals (*p<0.001*).

Figure 3: Changes in hemolymph protein concentration when *Triops* were transferred to different environmental Po2's. (A) Normoxic-reared females transferred to hypoxia. Pooled samples from 1-3 and 4-7 days after hypoxic transfer (B) Hypoxic-reared females transferred to normoxia. Pooled samples from 1-6 and 7-14 days after normoxic transfer (*p<0.05*).

Figure 4: Representative one-dimensional gels of changes in Hb subunit expression with time spent in either a normoxic or hypoxic environment. (A) Variation in Hb isoforms in normoxic-reared control (NC), hypoxic-reared control (HC) and hypoxic transferred animals (HTD = hypoxic transfer day (n)). (B) Variation in Hb isoforms in NC, HC and normoxic transferred animals (NTD = normoxic transfer day (n)).
Figure 5: ImageQuant Analysis of changes in Hb subunit isoforms in animals reared in normoxia and transferred to hypoxia. The intensity of each band (Hbα, Hbβ, Hbδ and Hbγ) is expressed as a percentage of the total intensity of all the bands in each lane. A: Hbα; B: Hbβ; C: Hbδ; and D: Hbγ. * Indicates significantly different from normoxic-reared control (NC) and hypoxic transfer day 1 (HTD1) (p<0.001).

Figure 6: Two dimensional gel electrophoresis of the hypoxic induction of Triops Hb. Normoxic-reared (NR), hypoxic transfer day 2 and 4 (HTD2, HTD4) and hypoxic-reared (HR). A train of spots corresponded with the molecular weights of Hbα and Hbδ therefore, more than one of the spots was removed and sequenced for comparison. The lettered circled spots were selected for NH₂-terminal amino acid sequencing.

Figure 7: (A) An example of a 2D DIGE gel obtained by combining samples of normoxic and hypoxic hemoglobin on the same gel. The gel was scanned at the appropriate wavelength for each fluor. Top panel, combined scan; lower two panels are the separated scans. (B) An example of DeCyder analysis of spots. Areas outlined in blue represent an increase of at least 2.5 times normoxic values. The spot enclosed in pink is the specific area being analyzed by DeCyder software. Lower panel demonstrates the difference in volume between the spot on the normoxic vs. hypoxic gel.
Figure 1

![Absorbance vs. Elution Volume Plot]

- ● Values 280
- □ Values 415

Absorbance

Elution Volume (ml)

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Figure 2

![Bar graph showing hemolymph protein concentration (mg/ml) for Norm and Hyp rearing conditions. The graph indicates a significant difference (*) between the two conditions.]
Figure 3

A

Hemolymph protein concentration (mg/ml)

Control  TD 1-3  TD 4-7

Days of Hypoxia

B

Hemolymph protein concentration (mg/ml)

Control  TD 1-6  TD 7-14

Days of Normoxia
Table 1: NH2-terminal amino acid sequences of Hb chains isolated from two dimensional gels. Spots were sequenced from both normoxic and hypoxic-reared animals. Spots are labeled in figure 6. If more than one amino acid was detected at the same position, they are shown with slashes in between. ND, not determined.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Amino acid(s) at position:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16</td>
</tr>
<tr>
<td>Norm Hbα 2</td>
<td>H P E C A G D S V V V T E T R/M/T</td>
</tr>
<tr>
<td>Norm Hbα 4</td>
<td>H R/P E C A/T G D S/N V V V T E T R T</td>
</tr>
<tr>
<td>Norm Hbβ</td>
<td>G D C G D S V V V T E T R T</td>
</tr>
<tr>
<td>Hyp Hbα 2</td>
<td>H P E C A G D S V V V T ND T R ND</td>
</tr>
<tr>
<td>Hyp Hbα 5</td>
<td>H R E C T G D S V V V T E ND R ND</td>
</tr>
<tr>
<td>Hyp Hbδ 1</td>
<td>G R L/G D S/Q V V/L V T E ND ND ND</td>
</tr>
<tr>
<td>Hyp Hbδ 3</td>
<td>G R L/G D S V V V T/I E ND ND ND</td>
</tr>
</tbody>
</table>
CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

General conclusion and relevance of the work

The results of this dissertation continue to reveal the complexity of the crustacean cardiovascular system. Hypoxia provides a tool for enhancing our understanding of the underlying mechanisms by which these animals are able to modify their responses in times of stress.

With the renewed interest in the metabolic demand of parental care, in particular brood care, in marine invertebrates, the work presented in Chapter 2 establishes that ovigerous *P. pugio* exhibit brooding behavior in the form of pleopod fanning that is enhanced during times of hypoxic stress. The observed increase in cardiac output with advanced reproductive states supports the hypothesis that gravid and ovigerous/gravid females incur greater metabolic demands than non-gravid females due to egg production and/or brooding behavior. Although gravid and ovigerous/gravid females were more sensitive to declining Po2, the lack of interaction between reproductive state and Po2 indicates that reproductively active females of *P. pugio* are well adapted to hypoxic stress and respond to hypoxic stress in a manner similar to non-gravid females.
The results from Chapter 2 led us to examine additional mechanisms by which gravid females are able to endure even severe hypoxic stress, namely changes in flow throughout the vascular system. Regardless of species or reproductive state, the data in Chapter 3 in conjunction with previous studies suggests that decapod crustaceans, including the gravid grass shrimp, require enhanced oxygen delivery to the sternal artery. Survival depends on the ability to enhance oxygen uptake. Increased flow through the sternal artery enhances hemolymph and oxygen delivery to the respiratory apparatus and the gills and as well as the ventral nerve cord and other nervous tissue. While a proportionately large amount of flow is directed toward the anterior lateral arteries and hence the ovaries, ultimately, the ability of the female to survive the hypoxic stress requires an increase in flow to vital areas such as the respiratory apparatus and nervous tissue via the sternal artery. Interestingly, the changes in flow occur at levels of hypoxia well above the hypoxic level that causes a fall in cardiac output. This suggests that the re-direction of flow, like ventilation, is an early response to a change in oxygen tension.

In the previous chapters there are no measures of intra-cardiac pressure, a key cardiac parameter. Additionally, the coupling of pressure and volume would allow for an assessment of stroke work (metabolism) of the heart. Since pressure-volume loops have not yet been used in an open circulatory system, the data presented in Chapter 4 demonstrates that single ventricle of *P. pugio* functions in a manner similar to the ventricles of animals with closed systems. The P-A loop of the grass shrimp has the same four primary phases as P-A loops
generated from the ventricle of a closed system: (1) iso-volmic contraction (2) ventricular emptying (3) iso-volmic relaxation and (4) ventricular filling.

Armed with this new tool we sought to test, in Chapter 5, alterations in cardiac mechanics during exposure to hypoxia. The work in Chapter 5 demonstrates two key points: 1) that intra-cardiac pressure falls and 2) that stroke work is reduced, upon exposure to hypoxia. Changes in resistance and flow throughout the vasculature may account for the fall in intra-cardiac pressure. It is the fall in intra-cardiac pressure that results in a fall in stroke work during hypoxia, so that the heart is working more efficiently, using less oxygen, during times of hypoxic stress.

Based on the evidence from the first four studies, the cardiovascular system is well adapted for handling hypoxic stress and does so by re-directing flow, slowing heart rate, dropping intra-cardiac pressure and ultimately reducing myocardial O\textsubscript{2} consumption. Alterations in oxygen binding pigments provide another additional, powerful mechanism for enhancing survival during hypoxic stress. In Chapter 6 we examine the role of altered oxygen binding pigment concentration and structure as an additional mechanism for enhancing survival during hypoxic stress.

The data from Chapter 6 clearly demonstrates that both developing and adult \textit{T. longicaudatus} respond to hypoxia by altering Hb concentration and subunit composition. The response to hypoxia can be induced in adulthood and is plastic during development. The plasticity of the response during development is permanent as we were unable to reverse the response in adults.
Taken together the results of these studies indicate that life history stage must be taken into consideration when examining the response of the cardiovascular system to stress. Ultimately, the use of new tools like pressure-area loops will further enhance our understanding of cardiac mechanics and energetics during times of stress. Finally, alterations in oxygen binding pigments provide a link between the cardiovascular delivery system and the mechanism of enhanced oxygen uptake and delivery.

Future directions

The data presented in Chapters 4 and 5 establish the use of P-A loops in the study of cardiac mechanics and energetics in the single ventricle of decapod crustaceans. In Chapters 2 and 3 we explore the impact of hypoxia on the cardiovascular system of reproductively active females. These studies can be enhanced with the use of P-A loops to examine changes in cardiac mechanics during various reproductive stages. The P-A loops that have been used extensively to the study developmental and functional aspects of the four chambered heart (for review see Burkhoff et al., 2005) can now be used to enhance our understanding of cardiac mechanics during development and stress. A complex system of neurohormones affect cardiac function in crustaceans, such as control of heart rate, stroke volume and flow (Saver and Wilkens, 1998; Wilkens, 1999); however, teasing apart the precise location of action has been difficult. The use of P-A loops can enhance our understanding of how these neurohormones alter cardiac contraction and hence cardiac
mechanics. Overall, the advent of P-A use in the ventricles of open circulatory systems will provide new insights into yet unexplored areas of cardiac function.

The alterations in oxygen binding pigment concentration and subunit composition demonstrated for Triops in Chapter 6 open the door for continued study. Little is know about Hb or Hc turnover rate and regulation. The unique finding that Triops Hb was not down regulated when hypoxic reared adults were retuned to normoxia leads to further questions about the location and regulation of synthesis and degradation of oxygen binding pigments. Hemocyanin synthesis occurs in the hepatopancreas of decapod crustaceans (Durstewitz and Terwilliger, 1997) and Hb synthesis is thought to occur in the fat cells and epipodites of branchiopod crustaceans (Goldmann, 1999). In neither case have there been studies on the location and regulation of degradation of oxygen binding pigments. The recent discovery of hypoxia inducible factor in the branchiopod crustacean Daphnia magna (Gorr et al., 2004) and the decapod crustacean Cancer magister (Head and Terwilliger, 2004) will provide additional avenues for the future study of both Hb and Hc regulation at the genetic level.
References


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Publications

Guadagnoli, J.A. Tobita, K. and Reiber, C.L. (in prep) Using the pressure-area relationship to access changes in cardiac work during hypoxia in the open circulatory system of the grass shrimp, *Paleamonetes pugio*. (*American Journal of Physiology: Integrative and comparative physiology*)


Dissertation Title: Short-term compensatory and long-term plastic cardiorespiratory responses to hypoxic exposure

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