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The Grass Shrimp, Palaemonetes, Pugio: Hypoxic Influences on Embryonic Development

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THE GRASS SHRIMP, *PALAEMONETES, PUGIO*: HYPOXIC INFLUENCES ON EMBRYONIC DEVELOPMENT

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GENERAL ABSTRACT

Grass shrimp, *Palaemonetes pugio,* can survive in brackish waters and estuarine ecosystems despite the frequent oscillations and fluctuations in salinity, temperature and oxygen. Adult *P. pugio* have the ability to osmoregulate (Romney and Reiber 2011), change cardiac parameters to tolerate temperatures (not yet published, Mika and Reiber) and oxyregulate (Guadagnoli and Reiber 2013). Manipulation of cardiac parameters allows for these methods of regulation. However, cardiac contraction and internal convection of oxygen do not occur until later stages of embryonic development. Studies focused on these morphological and physiological advantages may provide further understanding of the regulatory mechanisms within grass shrimp embryos, larvae and adults. To answer these questions, experiments are conducted under controlled laboratory conditions. The purpose of this study is to examine the effects of varying oxygen conditions on *Palaemonetes pugio*, a brackish water crustacean. Specifically, the study determines whether developmental and physiological changes contribute to increased survivorship of *P. pugio* embryos. Changes in rate of embryonic oxygen consumption under normoxic conditions during cardiac development will be compared with oxygen consumption rates under hypoxic conditions to quantify any changes in oxygen uptake. Furthermore, we will determine if exposure to variable oxygen conditions influences metabolic processes, whether oxyconformation shifts to oxyregulation and finally, quantify the amount of lactate production per *P. pugio* clutch.

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CHAPTER 1 INTRODUCTION

The grass shrimp, *Palaemonetes pugio*

Paleomonetes pugio, otherwise known as grass shrimp, serve as an ideal model organism for examination of cardiovascular physiological development. Similar to the translucent, protective chitin of adult *P. pugio,* embryonic grass shrimp possess a clear chorion which allows for non-invasive observation of cardiac development. Adult morphology and physiological parameters have been previously described; however, embryonic grass shrimp description of physiological parameters remains vague. An embryonic staging scheme has recently been established which dictates cardiac development throughout specific stages, initiation of intermittent cardiac contraction and finally, achievement of steady cardiac beats (Romney, 2010). Temporal development and physiological changes under experimental conditions have not yet been conducted on embryos.

Adult and embryonic animals alike are easy to maintain under laboratory conditions, as natural environmental factors can be simulated. The breeding season spans from February through October (Anderson, 1985). Within this time frame, numerous ovigerous females carry large clutches of eggs. An ovigerous female carries clutch sizes ranging from 200 to 400 eggs, thus provides an abundant sample size and decreases confounding effects based on variation within individuals (Alon and Stancyk, 1982). Fertilized embryos are carried externally along the pleiopods of the female and adhere by attachment to setae. Exposure to fluctuating environmental conditions is readily witnessed in the externally developing embryos (Bauer and Abdalla, 2000). Despite

extreme changes in oxygen levels, salinity and temperature, grass shrimp adults and embryos persist in ample numbers within their ecological niche.

Paleomonetes pugio serve as an ideal model organism for examination of cardiovascular physiological development. Similar to the translucent, protective chitin of adult *P. pugio,* embryonic grass shrimp possess a clear chorion which allows for non-invasive observation of cardiac development. Adult *P. pugio* have morphological advantages that allow for regulation of oxygen through internal convection processes (Guadagnoli and Reiber 2007); however, embryonic grass shrimp description of physiological parameters remains vague. No previous studies have detailed embryonic oxygen consumption ($\dot{M}O_2$) in a closed, normoxic system. Even more, no previous studies have accounted for embryonic $\dot{M}O_2$ in a closed, hypoxic system. Normoxic $\dot{M}O_2$ will be calculated for each stage of development under controlled laboratory conditions. Changes in rate of embryonic $\dot{M}O_2$ under experimental hypoxic conditions will be compared against the normoxic baseline. Specifically, we will determine if exposure to variable oxygen conditions influences metabolic processes, whether oxyconformation shifts to oxyregulation and finally, quantify the amount of lactate production per *P. pugio* clutch. The purpose of this thesis is to provide a thorough descriptive assessment of metabolic processes that occur under various oxygen conditions throughout *P. pugio* embryonic development.

The compensatory mechanisms exhibited by *P. pugio* embryos have not been explored. Further understanding in this area may provide a physiological basis of regulatory

mechanisms within grass shrimp embryo development. According to prior studies, embryos have not been shown to possess the ability to manipulate oxygen regulation parameters until later stages in embryonic development (Romney and Reiber 2011). Despite this, clutches hatch in abundant numbers throughout the breeding season. An embryonic staging scheme has recently been established which describes cardiac development throughout specific stages, including the initiation of intermittent cardiac contraction and finally, achievement of steady cardiac beats (Romney and Reiber 2011). Each stage has been described on an anatomical, morphological, volumetric and cardiac physiological basis, but has not delved into metabolic processes involved in development. Studies conducted throughout this thesis will use the established staging scheme to determine physiological and metabolic changes under experimental oxygen conditions in *P. pugio* embryos. We wish to explore the processes involved in embryo survival upon development under low oxygen conditions. The model organisms of this study are *Palemonetes pugio* collected from the Gulf Coast and raised in 20 gallon (75.7 L) tanks at 21˚C, 30 ppt and exposed to a natural photoperiod (12L:12D).

Animal exposure to fluctuating oxygen conditions

Palaemonetes pugio, grass shrimp, persist among diverse ecological niches, ranging from brackish waters to saltwater ecosystems (Alon and Stancyk 1982). *P. pugio* are abundant along the Atlantic and Gulf Coasts of North America, predominantly in estuarine environments with highly variable conditions. Estuarine systems are characterized by frequent fluctuations in several ecological parameters that may affect physiological homeostasis and create a dynamic environment for the inhabitants (Welsh 1975). Despite

these fluctuations, *P. pugio* withstand drastic changes throughout a daily cycle. They effectively live and breed within these environments that may range in dissolved oxygen levels from 4 to 22 kPa O_2, salinities of between 0 to 55 parts per thousand (seawater at 30-32 parts per thousand; ppt) and temperatures of 5˚ to 38˚C (Anderson 1985; Brown-Peterson *et al*. 2008; Cochran and Burnett 1996; Knowlton and Kirby 1984; Welsh 1975).

Frequent exposure to oscillating oxygen levels results in an animal's need to regulate internal physiological parameters and maintain homeostasis. This need to maintain homeostasis is heightened in animals inhabiting estuarine environments; hence, the need for regulation. Attention has been directed towards physiological mechanisms that underlie maintenance of hemolymph oxygen levels. It has been demonstrated that aerobic metabolism and regulation is intricately involved in this mechanism (Guadagnoli and Reiber 2011). Direct measurements of cardiovascular parameters: stroke volume, heart rate and subsequent calculation of cardiac output, hemolymph flow and Pressure-Area response as a whole describe the physiological correlation to these environmental changes (Harper and Reiber 2004; Guadagnoli and Reiber 2011). To further our understanding of the physiological effects of hypoxia on both adult and embryonic crustaceans, one must first become familiar with cardiovascular anatomy as it develops from the embryo, to larva, and to the finalized adult heart structure.

Cardiac development in crustaceans

The bilaterian body plan is highly conserved between vertebrates and invertebrates (DiRobertis 2008). Bilateral pattern encompasses the cardiovascular systems of a majority of organisms as well (Olsen 2006). Arthropod cardiac development has been well studied in insects such as *Drosophila melanogaster,* the fruit fly. However, development of the heart in aquatic organisms has not been described in depth. Research on crustacean cardiac development can help to elucidate the effects of highly variable aquatic environments on crustacean embryos, which are directly exposed to extreme temperature changes, oxygen levels and salinity gradients throughout development (Welsh 1975; Torres *et al.* 2007; Brouwer 2008; Li and Brouwer 2009; Guadagnoli and Reiber 2011). Recently, there has been growing interest in aquatic animal cardiac development. An integrative scientific approach combining developmental, molecular, physiological and evolutionary theories provides a more comprehensive viewpoint and contributes to the under-studied sector of crustacean cardiac development. This section will review newly published research describing crustacean cardiac development beginning from cell lineage tracing of multipotent mesoderm cells through terminally differentiated cardiac cells.

In several crustacean species, external fertilization occurs when the male deposits spermatophore to the oviduct of the female. In the oviduct, unfertilized eggs move from the ovary towards the spermatophore. The eggs become fertilized and form a single-cell stage zygote (Welsh 1975). The zygote then undergoes cleavage to increase number of cells, while embryonic volume does not significantly increase (Welsh 1975; Romney and

Reiber 2011). Gastrulation then involves formation of 3 germ layers and appears highly conserved among crayfish taxa (Vilpoux *et al.* 2006). Succeeding stages of crustacean embryo development generate muscle precursor cells. This process has been described in *Drosophila melanogaster* and has become accepted in crustacean research as well. The current view of this cell model proposes that adult muscle groups initially form along the anterior-posterior axis during crayfish embryogenesis (Price and Patel 2008; Jirikowski 2010). Muscle pioneer cells along the anterior-posterior axis recruit undifferentiated mesoderm cells and fuse to shape the scaffold for multicellular syncytial muscle precursors (Paululat *et al.* 1999; Xavier-Neto 2012). Mononucleate muscle pioneer cells elongate to form F-actin fibers that interact with myosin-HC, creating the striated sarcomere structure of cardiac mesoderm similarly seen in *Drosophila*. In arthropods, the heart originates from the dorsal mesoderm, providing strong evidence that cardiac formation processes are conserved among insects and crustaceans (Janssen 2008; Jirikowski 2010; Hunnekuhl and Wolff 2012). In the marbled crayfish, *Procambarus fallux,* progenitor mesoderm cells give rise to trunk mesoderm from which several muscle groups arise and myocardiocytes will originate (Jirikowski 2010). Characterization of F-actin myofibril isoforms among specific muscle cells depends on function and spatial orientation in the developing embryo (B.W. Kim *et al* 2009; Jirikowski 2010). Among these isoforms, a cardiomyocyte F-actin isoform was identified, stained with phalloidin and followed through embryonic stages 5 to 10 (described in Table 1). The processes of cardiac development described in the marbled crayfish are consistent with processes occurring in grass shrimp embryos (BW Kim 2009; Jirikowski 2010; Romney and Reiber 2011).

Initial cardiac contractions are observed in *P. pugio* embryonic stage VIb, followed by stronger, coordinated contractions prior to hatching (Jirikowski 2010; Romney and Reiber 2011). Similarly, in the amphipod crustacean *Orchestia cavimana*, functional cardiac contraction appears at stage 6 when the thoracic musculature has differentiated (Hunnekuhl and Wolff 2012).

Based on this evidence, embryonic stages preceding initiation of cardiac contractions may be in the process of creating the thoracic scaffold and organizing mesodermal precursors. Further studies must be conducted to support this hypothesis.

Table 1. F-actin Related Cardiogenesis in the Marbled Crayfish*

Embryonic Stage	Developmental Feature
5	Initial appearance of heart-forming cell isoform on dorsal side
6	F-actin heart primordium exhibits irregular contractions
7	Contractile heart membrane expands anterio-posteriorly and dorsally
8	Dorsal extensions of the contractile network form the tubular heart
9	Myocardium becomes dense and ostia become visible
10	Alary muscles become visible on the ventral myocardial surface

** adapted from Jirikowski 2010 and BW Kim 2009*

Following thoracic scaffold formation in both crayfish and lobster embryos, larval hearts possess a tubular morphology (Figure 1 A); ultimately, adult hearts acquire a bulbous shape (Figure 1 B) (Wirkner and Richter 2009). In both crayfish and lobsters, prospective anterior aortic cells, otherwise defined as longitudinal muscle strands, develop into the anterior aortic vessel. This transition from heart to aortic specification

results in the complete loss of detection of myosin-HC signal, which is specifically detected in the tubular larval heart (Jirikowski 2010). Consistent with the founder cell model, the myosin-HC signal is detected only in early embryonic stages during which cells involved in tubular heart formation are recruited to the thoracic scaffold. Interestingly, the myosin-HC signal is never detected along the presumptive posterior aortic region, further suggesting that early myosin-HC isoforms in the anterior aortic region recruit cells for formation of the primitive tubular heart (Wilkens 1999; Wirkner and Richter 2009; Jirikowski 2010). Mesodermal cell fates are established at an early time point during the segmentation process (Price and Patel 2008). Studies using *Macrobrachium rosenbergii* indicate a model showing further evidence that cardiac muscle originates from the mesoderm and migrates into a saclike region of the heart (Pakeendarong 2010). Studies conducted on the amphipod crustacean *O. cavimana* give more insight that this predetermination may be attributed to spatial arrangement along the dorso-ventral axis (Hunnekuhl and Wolff 2012). After mesoderm precursor segments have been established, tubular embryonic and larval heart morphology is arranged (Figure 1A) (Hunnekuhl and Wolff 2012). Adult crustacean hearts become bulbous and provide intrinsic hemolymph circulation (Figure 1B). This transition from tubular to bulbous morphology may be marked by initiation of neurogenic control from the cardiac ganglion. Though adequate hemolymph circulation persists in the early stages of heart development through myogenic control, maturation continues beyond larval development (Spicer 2001; McMahon 2008). Heart rate and stroke volume in adult crustaceans function under neurogenic control from the cardiac ganglion and the central nervous system (Romney and Reiber 2011). Complete growth of the bulbous adult heart and

excitatory effects produced by the cardiac ganglion do not occur until later juvenile stages. This is supported by experiments using injection with tetrodotoxin (TTX), a neurotoxin that blocks Na^+ voltage gated channels and prevents action potential firing (Spicer 2001; Hwang 2007). TTX injections throughout sequential juvenile stages of *Metapenaeus ensis*, the sand shrimp, led to the discovery that fatal effects occur only in juveniles weighing more than 25 milligrams, implying that nerve control is initiated during this transition (McMahon 2002). Further studies on the crab *Neohelice granulata* cardiac ganglion anatomy show similarities to other decapods in that neuronal processes ramify within the dorsal wall of the adult heart and provide an intrinsic "pacemaker" system to distribute neuronal signals to myocardiocytes with which the cardiac ganglion is entangled (Yang 2013). These data indicate that cardiac ganglion function is not fully developed until later juvenile stages and there is a transition from myogenic to neurogenic control in cardiac muscle during development. These complex cardiac events occur simultaneously throughout development of an entire grass shrimp clutch (approximately 200 embryos) (Romney and Reiber 2010). This temporal coordination is interesting because grass shrimp embryos are carried externally, exposed to extreme aquatic estuarine conditions, yet maintain synchronous embryoic developmental processes. Identification of the mechanisms used to maintain homeostasis throughout development in grass shrimp embryos will greatly contribute to studies regarding development in aquatic organisms.

Grass shrimp embryonic cardiac anatomy and physiology

Unlike most birds and reptiles, crustaceans carry developing embryos externally and have a higher tendency to drop the clutch if environmental conditions are too severe, thus abandoning parental investment. Due to these complications, crustaceans have evolved mechanisms to compensate for decreased maternal care periods. An embryo will molt its protective chorion once internal yolk has been depleted, marking the transition from embryonic to larval stage. Development of the cardiovascular system cues time of larval hatching (Romney and Reiber 2011). Once the circulatory system develops and metabolizing mass in the embryo increases, rate of yolk hydrolysis accelerates, implying an increase in metabolic rate (Habashy *et al.* 2012). Cardiac development and highly conserved events that occur during development allow for accurate assessment of processes occurring at each developmental time point.

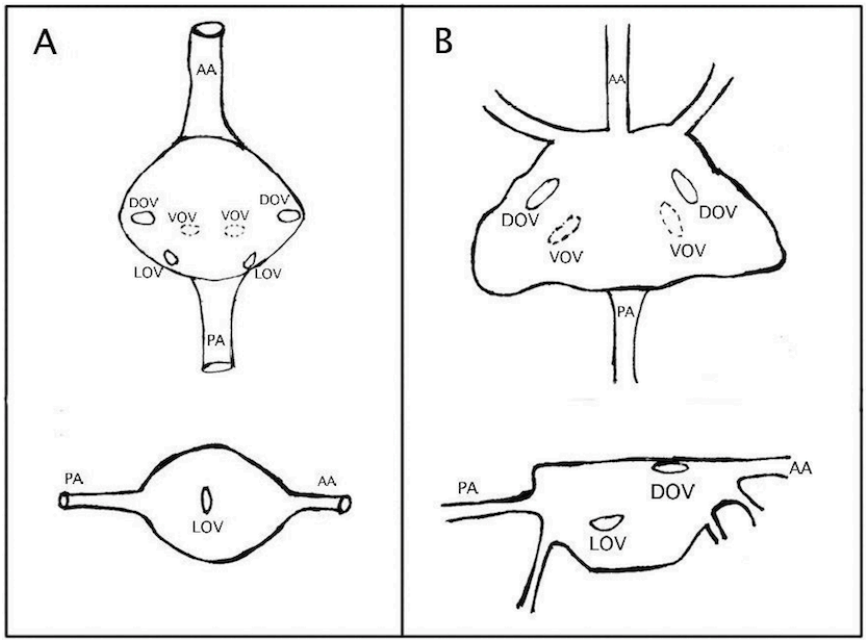

Figure 1. Embryonic Tubular Heart and Adult Bulbous Heart. Embryonic heart (left); adult heart (right).

Due to the conserved nature of bilaterian circulatory morphology, detection of specific cardiac markers serves as a major tool to compare and establish temporal staging schemes in non-model organisms. Even more, the transparent chorion of crustacean embryos provides a non-invasive method to obtain these observations. Despite this, few studies exist that delve into the characterization, development and molecular nature of crustacean embryos. The circulatory system is the first to fully develop during organogenesis (Harper and Reiber 2004). Coordinated myocardial contractions, formation of ostia pairs and alary ligaments are not apparent until development of the final adult segment (Spicer 2006). Embryonic staging schemes describing cardiac formation have been established in *Macrobrachium rosenbergii, Macrobrachium carcinus* freshwater prawns and *Palaemonetes pugio* (Lara and Wehrtmann 2009; Habashy *et al.* 2012; Romney and Reiber 2011). Developmental features between these species are highly conserved and most obviously perceived in the dorsal placement of the heart.

In addition to similar anatomical heart morphology, functional homologies arise in the crustacean embryo circulatory system and include: providing an internal conduction mechanism, serving as a hemolymph reservoir and aiding in regulation of stroke volume to allow acclimation to oscillating environments (Romney and Reiber 2011; Xavier-Neto 2012). Two highly evolved decapod crustaceans, *Metapenaeus ensis* and *Palaemonetes pugio*, possess short and globular heart morphology in larval stages (Figure 1A). During embryonic development in both species, initiation of myocardial contraction occurs during the naupliar stage when the previously established thoracic segments provide a

foundation to anchor the heart structure (Harper and Reiber 2004, Romney and Reiber 2011). Coincidentally, initiation of cardiac contractions is accompanied by an increased rate of yolk hydrolysis in *M. rosenbergii* (Habashy *et al.* 2012), possibly providing a physiological cue for the embryo to hatch to larval form and actively search for an external nutrient source to continue development. It is unknown whether oxygen regulation occurs in the crustacean embryo, studies described in this thesis will address this issue.

Grass shrimp adult cardiac anatomy and physiology

The heart of the adult *P. pugio*, like most decapod crustaceans, consists of one ventricle to pump oxygenated hemolymph through multiple arterial outlets, providing a singular flow entering and leaving the heart (Figure 1B). Multiple sets of ostia (3 sets) and aortic valves located at the entrance of each vessel provide intricate control of the hemolymph distribution and cardiac filling. The valves work in coordination to create a one-way flow of hemolymph through the heart—an important factor in organisms with open circulatory systems. Ostia receive hemolymph while aortic valves disperse oxygenated hemolymph throughout the systems. Pre-branchial, deoxygenated hemolymph from surrounding tissues is targeted towards the gills for reoxygenation. Post-branchial oxygenated hemolymph is then delivered through specified branchio-cardiac veins to the heart for re-distribution throughout the tissues. This one-way flow established by the single ventricle ensures hemolymph directionality and a constant cardiac cycle (Guadagnoli and Reiber 2007).

Neurogenic control of the heart provides *P. pugio's* main source of adult cardiac contraction. The central nervous system conducts action potentials to the dorsal cardiac ganglion, allowing excitatory or inhibitory response by the cardiac myocytes. Mammalian hearts are similarly controlled by an intrinsic nodal conduction system and possess a "pacemaker", but are also induced by the parasympathetic nervous system. Cardiac control by an intrinsic pacemaker suggests there may be parallels, indicating early evolutionary divergence between crustaceans and mammals.

The heart responds to environmental fluctuations accordingly. Here, we focus on effects of hypoxia on cardiac parameters in adult crustaceans. Several studies have been conducted to determine crustacean response to hypoxic conditions. Generally, larger crustaceans maintain steady cardiac output under hypoxia through a decrease in heart rate and increase in stroke volume (Reiber 1995). Harper and Reiber (2004) described adult *Palaemonetes pugio* response as an increase in heart rate and decrease stroke volume in order to maintain steady cardiac output from hypoxic ranges 13.3 kPa O_2 through 8.0 kPa O_2. Heart rate seemed to be the primary regulator of cardiac and oxygen homeostatis in *P. pugio*. However, recent studies have challenged this idea and rather support that hypoxia-induced bradycardia occurs in adult grass shrimp as well (Guadagnoli and Reiber 2011). In other words, to maintain cardiac output under hypoxic conditions, the adult grass shrimp will decrease heart rate to increase stroke volume, thus maintaining cardiac output (Guadagnoli and Reiber 2011). This mechanism provides an immediate means for adult grass shrimp to survive under fluctuating oxygen conditions and adjust as necessary.

Pressure-Volume and Pressure-Area loops have provided further evidence of oxygen regulation (Guadagnoli and Reiber 2011). The ability for *Palaemonetes pugio* grass shrimp to survive under extreme hypoxic conditions is attributed to the fact that cardiac work is significantly decreased. This decrease is accomplished by (1) hypoxia-induced-bradycardia and (2) a decrease in intra-cardiac pressure. Hypoxia-induced-bradycardia results in decreased heart rate, increase in stroke volume and maintenance of cardiac output. The decrease in intra-cardiac pressure resulting from decreased contractility and decrease in recoil of cardiac components, contributes to the increase in stroke volume. Together, these parameters decrease the overall cardiac work necessary to maintain adult grass shrimp cardiac output under hypoxic conditions.

Research objectives

Integration of various scientific approaches has produced a comprehensive snapshot of crustacean development. Descriptive analysis of cardiogenesis, study of cardiac physiology and cell lineage tracing in embryonic crustaceans all demonstrate the highly conserved nature of heart development and genetic homology between insects and crustaceans. Crustaceans endure external embryogenesis under extreme environmental gradients, have abundant clutches and possess a clear chorion, possibly making this organism a candidate invertebrate model. Further research into the development and physiology of grass shrimp may provide accurate temporal description of mesoderm scaffolding and organizing events, specification of transition from maternally driven development to independent zygotic transcription and the metabolic processes that accompany this development. Contributions from *Palaemonetes pugio* research may

illuminate questions about aquatic development, exposure to extreme environmental conditions and encourage a more integrative scientific method. The remainder of this study will focus on *P. pugio* embryonic development in aquatic systems ranging from normal to moderately severe oxygen availability.

The results from this study will shed light on the physiological and developmental compensatory mechanisms that occur in aquatic embryos upon exposure to a range of external oxygen concentrations. The first objective of this study is to determine oxygen consumption rates per stage of embryonic development under controlled laboratory conditions of 20˚ C, 12 hour light: 12 hour dark photoperiod, constant salinity of 30 parts per thousand (30 ppt), and starting oxygen conditions of 20.0 kPa O_2. This will allow extrapolation of the critical oxygen pressure (P_{crit}) per stage and delineate the shift from oxygen conformation to oxygen regulation in *Palaemonetes pugio* embryos. Second, we wish to determine oxygen consumption rates per stage of embryonic development under moderate hypoxic conditions (11.0 kPa O_2– 10.5 kPa O_2), maintaining the controlled laboratory conditions of 20˚ C, 12 hour light: 12 hour dark photoperiod, constant salinity of 30 parts per thousand (30 ppt). Here, we can also extrapolate the P_{crit} of each embryo under hypoxic conditions. Finally, we wish to assess whether a shift from aerobic metabolism to anaerobic metabolism occurs during any point of embryonic development. This will be determined through conduction of lactate assays on whole embryos.

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CHAPTER 2 OXYGEN CONSUMPTION OF THE EMBRYONIC GRASS SHRIMP, *Palaemonetes pugio,* (CRUSTACEA, DECAPODA) UNDER VARYING OXYGEN LEVELS

Abstract

Estuary ecosystems provide a challenging environment for animals to live. In spite of this, *Palaemonetes pugio* grass shrimp embryos develop in abundance during an annual breeding season that lasts from February through October. One clutch ranges from 200-400 embryos per ovigerous female. Fertilization, development and hatching occur synchronously within each clutch (Romney and Reiber 2011). Here, we investigate the oxygen consumption rate ($\dot{M}O_2$) of a single embryo in a closed system while maintaining control laboratory conditions of 20° C, seawater 20 ppt, normoxic water (PO_2=18.5 kPa O_2) and 12L:12:D photoperiod. Additionally, to examine effects of fluctuating oxygen conditions, hypoxic oxygen consumption rate of a single embryo will be investigated using the same control laboratory conditions. All oxygen consumption ($\dot{M}O_2$) data were obtained through use of the OxySense (OxySense® 4000B, OxySense®, Inc., Dallas, TX, USA) and analyzed through a customized Python program. These data will provide a quantitative basis for metabolic processes occuring throughout *P. pugio* embryonic development. We show that normoxic $\dot{M}O_2$ shows consistent trends with embryonic growth rate—previously quantified through calculation of cardiac parameters including cardiac output, heart rate, stroke volume and ratio of embryonic surface-area-to-volume

(Romney and Reiber 2011). The data also indicate that hypoxic exposure contributes to a significant increase in $\dot{M}O_2$ of grass shrimp embryos.

Numerous aquatic species have adapted different techniques to deal with oscillating oxygen conditions in estuarine ecosystems. The transition from an oxyconformer to an oxyregulator is a well-studied mechanism used by crustaceans and other aquatic organisms. This transition can be quantified by the identification of an animal's critical oxygen pressure (P_{crit}). Here, we show that *Palaemonetes pugio* embryos in early stages must rely on diffusive properties because they do not possess the internal capacity to circulate oxygen. Grass shrimp embryos in later stages, however, possess an internal convective system and begin to regulate oxygen distribution to deep metabolizing tissue. These data will help to understand how grass shrimp embryos are able to survive in abundance while developing in fluctuating oxygen conditions.

Introduction

According to the *Palaemonetes pugio* embryonic staging scheme described under normoxic conditions, embryonic growth and development is initially supported by diffusion of water and nutrients across the chorion in stages I through VIa (Romney and Reiber 2011). Upon reaching stage VIb, cardiac contractions are initiated and provide an internal convection system. On this basis, we hypothesize that rate of oxygen uptake from external sources contributes to rate of embryonic development in *Palaemonetes pugio*.

$H0_1$: *Palaemonetes pugio* embryonic oxygen consumption rate under normoxic conditions is not significantly different than oxygen consumption rate under hypoxic conditions.

H1: *Palaemonetes pugio* embryonic oxygen consumption rate under normoxic conditions is significantly different than oxygen consumption rate under hypoxic conditions.

$H0_2$: Critical oxygen pressure (P_{crit}) will remain consistent in early embryonic and later embryonic stages.

H2: Critical oxygen pressure (P_{crit}) is higher in early embryonic stages then decreases in later embryonic stages. Lower P_{crit} indicates the transition from oxyconformer to oxyregulator and accompanies initiation of cardiac contractions in *Palaemonets pugio* embryonic stage VIb.

Materials and methods

Animal care: Adult *P. pugio* were obtained from Gulf Specimen Marine Laboratories, Inc. (Panacea, FL, USA). Experimental animals were maintained in 40L aquaria in aerated artificial seawater (30-32 ppt at 20-25˚C) with a 12L: 12D photoperiod. Aquaria were maintained individually using filters with frequent water changes. Animals were fed a high protein marine fish flake to encourage ova production (Ocean Nutrition Formula Two Flakes). Conditions were maintained at 20° C, seawater 20 ppt, normoxic water (PO_2=20.5 kPa O_2) and 12L:12:D photoperiod.

Embryo collection: Ovigerous grass shrimp were attached to the flattened end of a wooden applicator stick on the lateral cephalothorax with cyanoacrylate glue. Embryos were removed from pleiopods of ovigerous females and transferred to nursery chambers located on a continuously rotating platform to mimic movements of an ovigerous female. Conditions were maintained at 20° C, seawater 20 ppt, normoxic water (PO_2=20.5 kPa O_2) and 12L:12:D photoperiod.

Oxygen consumption: A single embryo was selected from the clutch and staged using a stereo-microscope (Leica MZ12.5, McBain Instruments). The embryo was placed in a sealed chamber containing 0.1 ml of normoxic seawater (PO_2=20.5 kPa O_2, 20 ppt, 20° C). Changes in PO_2 were obtained using OxySense (OxySense® 4000B, OxySense®, Inc., Dallas, TX, USA) and plotted using Microsoft Excel 2011. Normoxic conditions were isolated to PO_2 ranges from 20.0 kPa O_2 through 18.0 kPa O_2. Hypoxic conditions were simulated using the same controlled laboratory conditions. Moderate hypoxia is defined between 11.0 kPa O_2 through 10.5 kPa O_2. The closed chamber allowed for self-induced hypoxia, as the embryo decreased oxygen availability through time. A maximum of 5 hours was allotted per data point to ensure that the embryo remained at the specified developmental stage. After data collection and exposure to experimental conditions, embryos were placed on a microbalance to obtain wet weight (0.0001 mg accuracy; Cahn 21 automatic electrobalance, Cahn Instruments Div., Ventron Corp., Cerritos, CA, USA).

Oxygen consumption rates: Using custom Python scripts, written by Christopher M. Hardy, Doctoral Candidate at the University of Nevada, Las Vegas, raw oxygen pressure data from the OxySense was converted to oxygen consumption rates ($\dot{M}O_2$). The program is devised to create a streamlined, unbiased analysis of normoxic and hypoxic conditions. Each data point was normalized per embryo by division by wet weight. Annotated custom python scripts are included in appendix.

Critical oxygen pressure (P_{crit}): P_{crit} was determined by piecewise linear regression analysis through a LINEST program on Microsoft Excel 2011. The workbook was obtained and revised according to oxygen pressure parameters from: "http://processtrends.com/toc_trend_analysis_with_Excel.htm#Segmented_-_Piecewise_Regression"

Statistical analysis: Means and standard deviations were calculated for each developmental stage (n=1-9) using normoxic oxygen consumption (18.5 kPa O_2 – 18.0 kPa O_2) and moderate hypoxia oxygen consumption (11.0 kPa O_2 – 10.5 kPa O_2). The overall effects of oxygen consumption throughout development due to various dependent variables, stage and oxygen condition, were studied using an analysis of variance (ANOVA) to account for effects of oxygen availability (JMP®, Version <*1.4*>. SAS Institute Inc., Cary, NC, 1989-2007). A Tukey post-hoc test was used for pairwise multiple comparisons where a significance of $P \leq 0.05$ was accepted to reject the null hypothesis.

Results

Mass: Embryonic stage VIII mass for a single embryo (0.242 mg ± 0.037) is significantly higher than stages I, II, IIIa, IV and V ($p = 0.001$, $n = 3 – 11$) (Romney and Reiber 2011). These values are listed below and shown in Figure 1.

Table 1. Embryonic Mass

Stage	Egg mass (mg)
I	0.103 ± 0.009[a]
II	0.100 ± 0.011[a]
IIIa	0.113 ± 0.007[a]
IIIb	0.143 ± 0.033
IV	0.109 ± 0.004[a]
V	0.119 ± 0.014
VIa	0.147 ± 0.006
VIb	0.142 ± 0.015
VIIa	0.139 ± 0.013
VIIb	0.172 ± 0.008
VIII	0.242 ± 0.037[b]

Oxygen consumption: Oxygen consumption rates were determined under normoxic conditions in a closed oxygen chamber with a starting PO_2 of 20.0 kPa O_2 (200 mBar O_2) maintained under controlled laboratory conditions of 20° C, seawater 20 ppt and 12D:12L photoperiod. Individual data points describe the average oxygen consumption per embryonic stage (n=1-9). Normoxic oxygen ranges were confined to 18.5 kPa O_2 through 18.0 kPa O_2 to bracket a specific normoxic range throughout all experimental stages. When raising embryos under normoxic conditions (18.5 kPa O_2 – 18.0 kPa O_2), there is no significant difference in oxygen consumption rate throughout individual embryonic stages (Figure 2). However, upon raising embryos under hypoxic conditions (11.0 kPa O_2 – 10.5 kPa O_2), there is a significant increase in hypoxic $\dot{M}O_2$ compared with normoxic $\dot{M}O_2$ ($p = 0.0294$) (Figure 4). To further investigate the increase in hypoxic $\dot{M}O_2$, individual embryonic stages were analyzed. Results indicate that there is a

significant increase in $\dot{M}O_2$ during embryonic stage IIIb when embryos are raised under hypoxic conditions ($p = 0.0169$) (Figure 3).

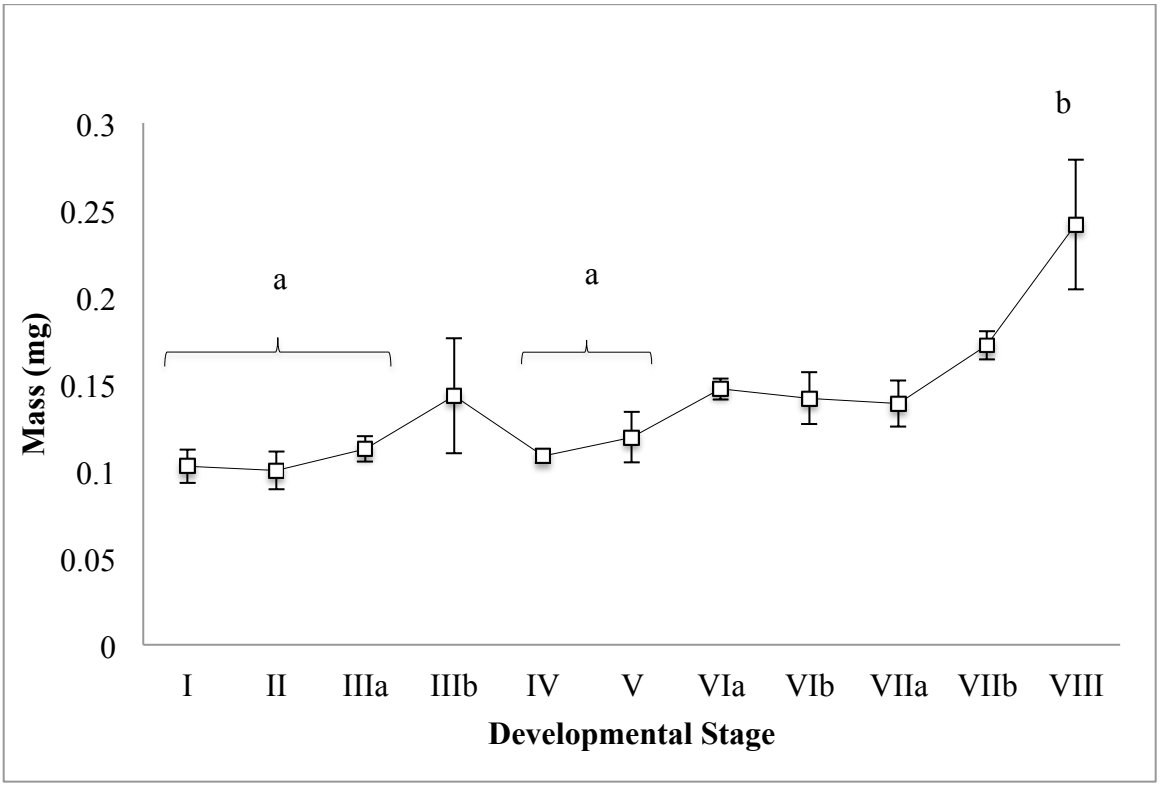

Figure 1. Embryonic Mass Versus Development in Normoxia (18.0 kPa O_2): Embryonic stage VIII mass for a single embryo is significantly higher than stages I, II, IIIa, IV and V ($p = 0.001$, $n = 3 – 11$).

Critical oxygen pressure results show a non-significant decrease throughout embryonic development (Figure 5). Though not significant, the decreasing trend is as predicted: Critical Oxygen Pressure decreases as the heart begins to gain more coordinated cardiac contractions. This further supports the idea that later stages can endure lower ambient oxygen levels and have transitioned from oxyconformation to oxyregulation. This is a

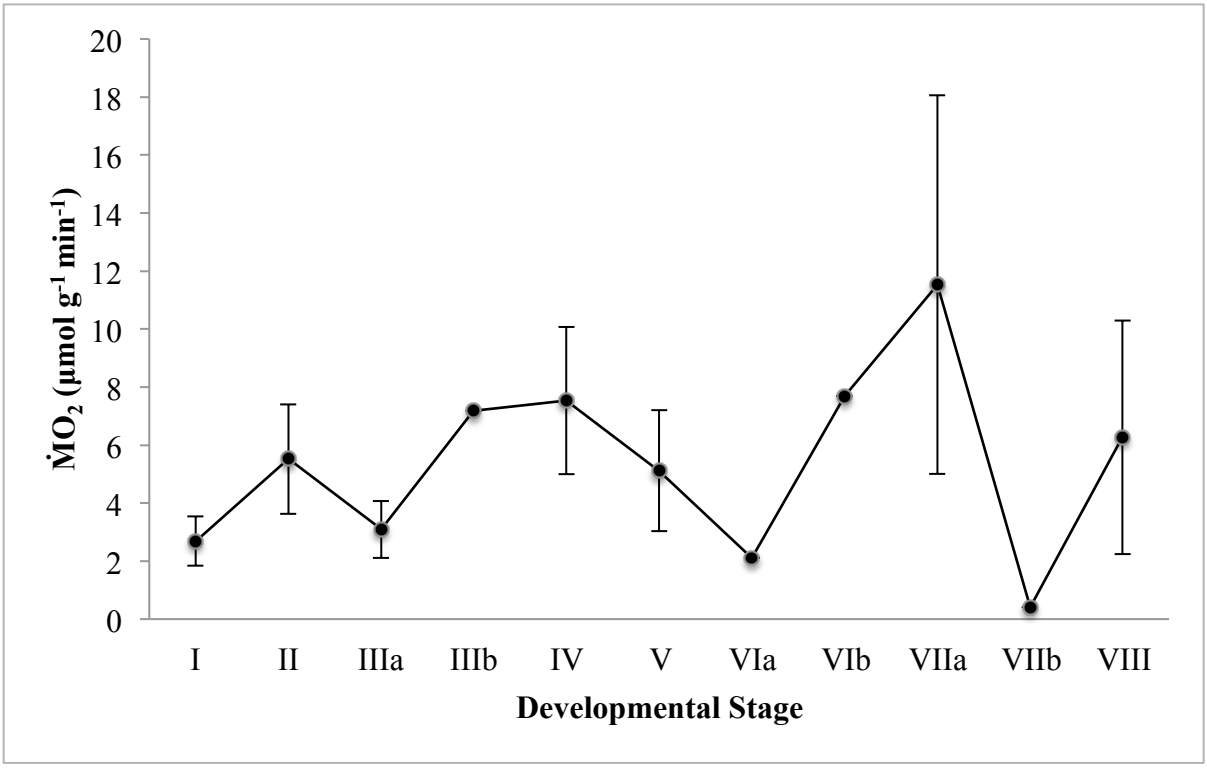

Figure 2. Average Normoxic $\dot{M}O_2$ per Embryonic Stage (18.5 kPa O_2 – 18.0 kPa O_2). Overall $\dot{M}O_2$ under normoxia undergoes no significant change between stages.

key physiological switch to those aquatic animals surviving in brackish, estuarine ecosystems.

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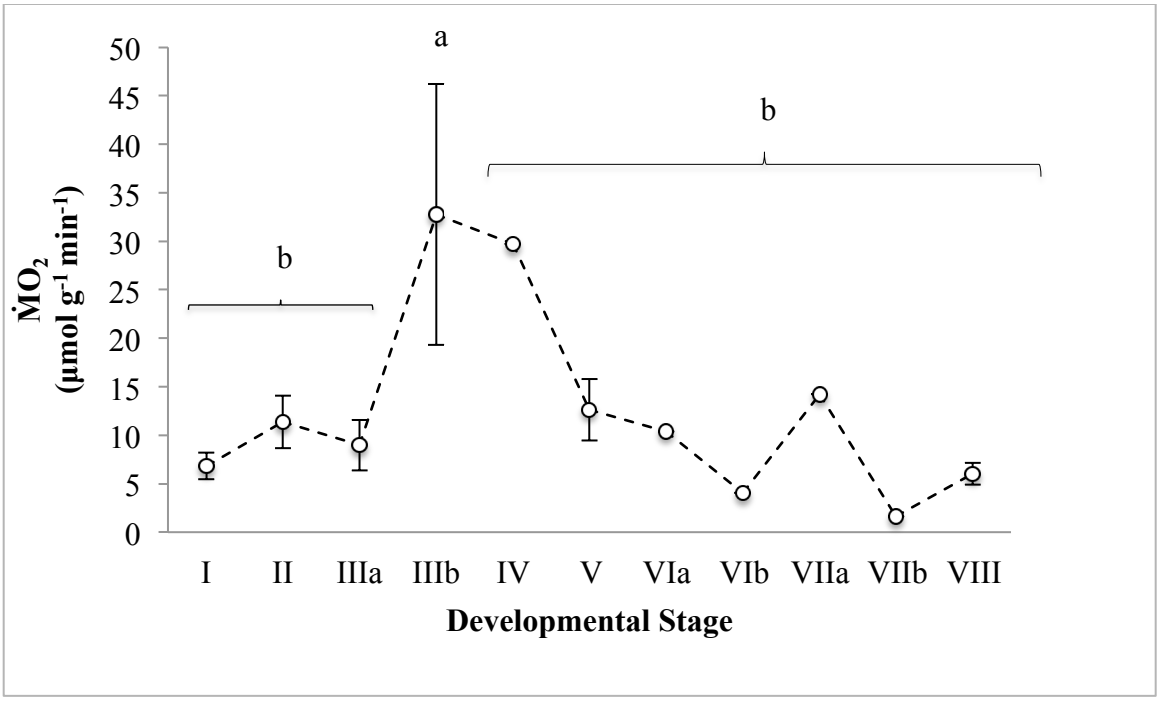

Figure 3. Average Hypoxic $\dot{M}O_2$ per Embryonic Stage (11.0 kPa O_2 – 10.5 kPa O_2). Embryonic stage IIIb undergoes a significant increase in $\dot{M}O_2$ under hypoxic conditions.

Critical oxygen pressure indicates the animal's tolerance to low oxygen conditions. P_{crit} represents the lowest oxygen level the embryo can be exposed, yet continue to function and develop normally. No previous studies have been conducted on grass shrimp embryos that analyze P_{crit} throughout development. We predict that tolerance of hypoxia will increase throughout embryonic development, as cardiac contractions become more coordinated and robust. As hypoxia tolerance increases, P_{crit} consequently decreases. In other words, embryos in later stages can tolerate lower oxygen pressure levels because the cardiac system has developed coordinated contractions, allowing for specific control of hemolymph flow and oxygen distribution throughout the animal.

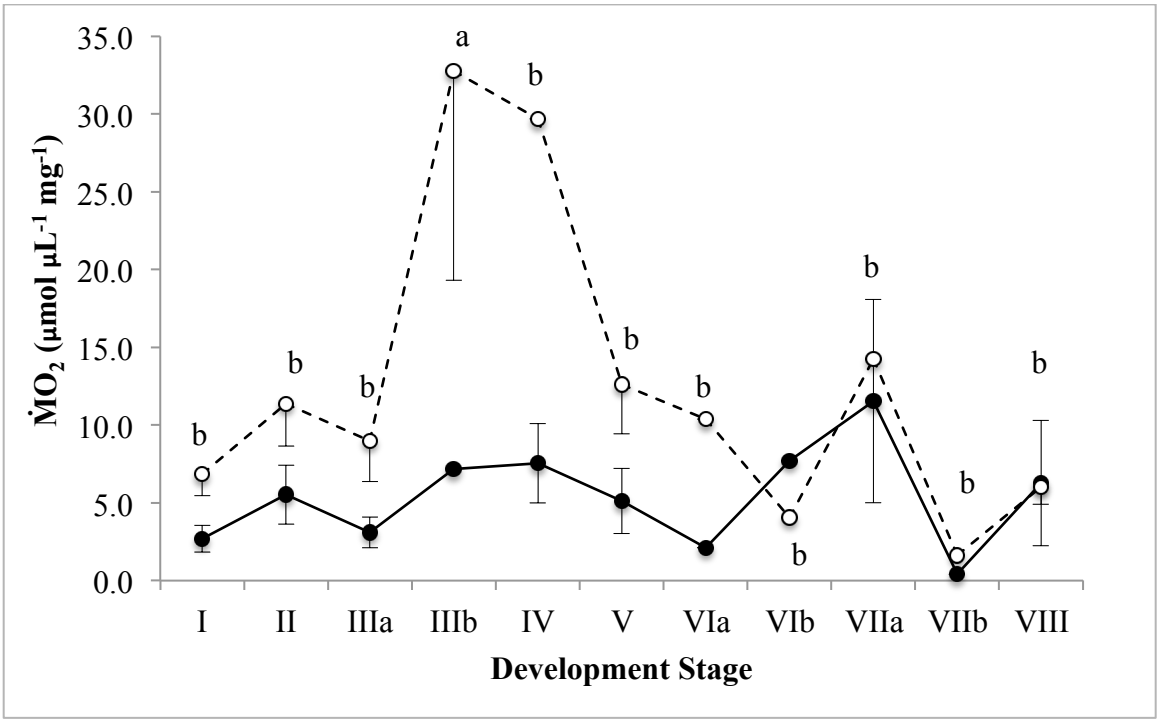

Figure 4: Normoxic Versus Hypoxic Oxygen Consumption Throughout Stages of Development. Oxygen consumption significantly increases upon exposure to hypoxia compared with normoxia ($p = 0.029$, $n = 1 – 9$). Specifically, $\dot{M}O_2$ significantly increases during embryonic stage IIIb ($p = 0.0169$, $n = 1 - 9$). There is no significant increase or decrease in embryonic $\dot{M}O_2$ between stages when embryos are raised under normoxic conditions. Solid line and closed circles indicate normoxic $\dot{M}O_2$; dotted lines and open circles indicate hypoxic $\dot{M}O_2$.

Analysis of oxygen consumption under two oxygen conditions, normoxia (18.5 kPa O_2 – 18.0 kPa O_2) and moderate hypoxia (11.0 kPa O_2 – 10.5 kPa O_2), supports that hypoxic conditions significantly increase oxygen consumption rates across embryonic developmental stages ($p = 0.0294$). Grass shrimp in estuarine ecosystems endure major oxygen fluctuations during a daily cycle (Welsh 1975). Despite this, grass shrimp adults and embryos alike continue survival in these estuaries.

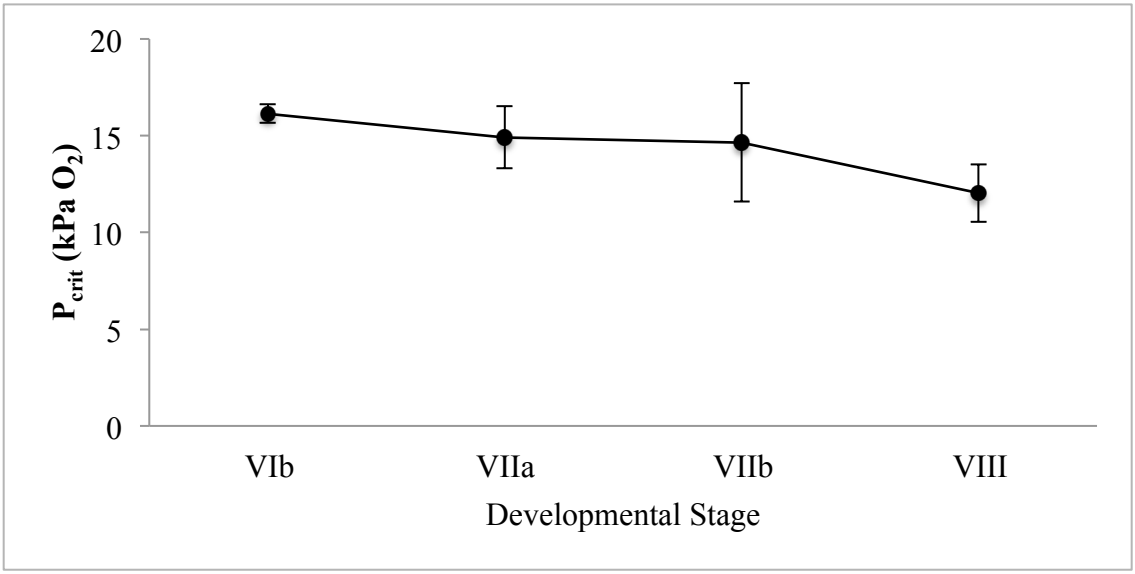

Figure 5. Critical Oxygen Pressure (P_{crit}) per Embryonic Stage. Embryonic stages VIb through VIII undergo a non-significant in P_{crit} during exposure to normoxic conditions (18.0 kPa O_2)

Discussion

An increase in oxygen consumption during exposure to hypoxic conditions suggests there is deviation from development in normoxia. It is possible that cardiac initiation occurs at an earlier time point when dissolved oxygen levels are decreased, or other compensatory mechanisms initiate at specific stages. Further analysis suggests that embryonic stage IIIb experiences a significantly higher hypoxic $\dot{M}O_2$ when compared with other embryonic stages raised in hypoxia ($p = 0.0169$) (Figure 3). This increase in $\dot{M}O_2$ suggests a developmental window at stage IIIb in which the embryo must activate compensatory mechanisms to continue with normal embryonic development under low dissolved oxygen conditions. The compensatory mechanisms in grass shrimp embryos are unknown and warrant further investigation into embryonic cardiac development,

analysis of cardiac parameters under hypoxic conditions and molecular analysis of genes that may undergo increased expression during hypoxic embryonic development.

An overall increase in hypoxic $\dot{M}O_2$, specifically during embryonic stage IIIb, may indicate that compensatory mechanisms are activated during this time to maintain homeostasis and continue with embryonic development. No previous studies in grass shrimp embryos have indicated an increase in oxygen consumption rate as a method of maintaining homeostasis during development in hypoxia. Adult grass shrimp studies support that upon exposure to hypoxia, the adult will decrease heart rate and increase stroke volume to maintain overall cardiac output (Guadagnoli and Reiber 2011). However, in Early and Gastrulation stages of embryonic development, cardiac contractions are not yet initiated (Romney and Reiber 2011) and therefore require other methods to compensate for a low oxygen environment. It is possible that internal, molecular mechanisms are activated during crucial points of embryonic development (Hand 2011).

P_{crit} serves as a strong determination as to whether the animal is an oxyconformer or oxyregulator (Richards 2011). A higher P_{crit} indicates the animal is an oxyconformer and relies largely on diffusive means to obtain oxygen from the environment (Richards 2011). A lower P_{crit} indicates that the animal is an oxyregulator and has other means of obtaining oxygen, such as internal convective processes or manipulation of cardiac contractions. *Palaemonetes pugio* embryos raised in normoxia do not possess the ability to regulate oxygen levels prior to stage VIb when cardiac precursors are in the process of

forming (Jirikowski 2010; Romney and Reiber 2011). Therefore, stages I through VIa are not included in this data set and are considered oxyconformers. Since our focus involves oxyregulation throughout development, data points obtained in this dataset were taken from embryonic stages VIb through VIII, when cardiac contractions have been initiated and the animal is a known oxyregulator. Upon raising embryonic grass shrimp in normoxic conditions, a non-significant decreasing trend in P_{crit} indicates there is no ongoing regulation to maintain oxygen consumption within the embryo (Figure 5). The non-significant decrease, is however, consistent with our hypothesis that as cardiac contractions become more coordinated, the animal can tolerate lower ambient oxygen conditions.

Our current studies have opened up the aquatic embryonic field to numerous interesting questions. Future studies in this area should attempt to further identify the specific level of hypoxia that is tolerable until the embryo can no longer proceed through development normally. This may be done by raising embryos in serial hypoxic conditions starting from normoxia and decreasing oxygen levels slightly, until critical oxygen pressure is obtained and compensatory mechanisms can no longer function. Finally, using an integrative approach, future studies may use molecular techniques to isolate proteins expressed during stressful environmental conditions. Such proteins include hypoxia-inducible factor-1 (HIF-1) and pyruvate dehydrogenase complex, which are upregulated and downregulated, respectively, during hypoxic exposure (Hand 2011). These proteins may provide insight as to what specific compensatory mechanisms allow for increased

oxygen consumption rates during embryonic development, especially for those stages prior to cardiac contraction.

Additionally, P_{crit} has not yet been determined in grass shrimp embryos raised in hypoxia. The non-significant decreasing trend in P_{crit} observed in our studies may be further amplified once the embryos are exposed to lower oxygen conditions. This can be accomplished in the same protocol that was used to collect P_{crit} data under normoxic conditions.

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CHAPTER 3 ANAEROBIC METABOLISM IN THE GRASS SHRIMP, *Palaemonetes pugio,* EMBRYO

Abstract

Anaerobic metabolism is the last ditch effort for animals to continue basal metabolic processes. Adult *Palaemonetes pugio* do not undergo anaerobic metabolism under hypoxia and high temperatures (Schofield and Mika 2011). No studies in anaerobic metabolism have been conducted on *P. pugio* embryos. Results from this study indicate that, when quantifying lactate accumulation before and after initiation of cardiac contraction, there is a significant decrease in lactate in the embryo. Cardiac contractions are initiated in the embryo at stages VIb. Lactate concentration significantly decreases at stage VIb, VIIb and VIII ($p = 0.001$, $n = 3 – 6$, $n = 1$ clutch of 100 – 200 embryos).

Introduction

Embryonic adaptations occuring as a result of exposure to environmental insults have accumulated numerous types of compensatory mechanisms. In crustaceans and insects, these mechanisms include diapause, sporulation, upregulation of hypoxia-inducible factor-1 (HIF-1) and downregulation of pyruvate dehydrogenase complex (Hand 2011). *Triops longicaudatus,* the horseshoe crab, may enter a sporulation stage; hatching in the larval form will occur only when abundant food sources become available. Upon hatching, the larva will undergo organogenesis and transition to complete adult form (Gilbert 2014).

Hindrances to successful hatching and survival in aquatic organisms include exposure to low oxygen conditions, extreme salinity gradients or extreme temperatures (Welsh 1975). *Artemia fransciscana* embryos, decapod crustaceans, suffer DNA damage when exposed to prolonged bouts of anoxia (McLennan 2009). Our model organism the grass shrimp, *Palaemonetes pugio,* is a brackish water inhabitant and endures harsh fluctuations in a daily bout (Welsh 1975). Even more, grass shrimp embryos develop externally and delicate organogenesis processes occur upon exposure to the same oscillating conditions which adult grass shrimp are exposed. Despite these obstacles, *P. pugio* remain an abundant species within brackish waters. Large clutches of embryos carried externally are able to survive from fertilization to hatching, thus ensuring the propagation of this species. Our data show that embryonic development continues to occur even under moderate hypoxia (11.0 kPa O_2– 10.5 kPa O_2). However, it is unknown whether the developing embryo enters an anoxic state. To test this, we quantify lactate accumulation in several *P. pugio* clutches where each clutch contains 200 – 400 individual embryos (Romney and Reiber 2011). Lactate quantification in the embryo serves as a viable indicator of amount and/or duration of hypoxia the individual has been exposed (Bridges 1979). From these data, we also determine if embryonic development in *P. pugio* relies on anaerobic metabolism at any point from fertilization to hatching.

Though it has been shown that embryos use compensatory mechanisms to survive moderate hypoxia (11.0 kPa O_2 – 10.5 kPa O_2), no evidence of anoxic processes have been described. Therefore, we hypothesize the following:

H0: Anaerobic metabolism occurs during embryonic development. In early stages of development, diffusion of gases through the chorion are insufficient to support aerobic metabolism and growth of the embryo. Anaerobic metabolism must be activated in order for development to continue.

H1: Anaerobic metabolism does not occur during embryonic development. In early stages of development, diffusion of gases through the chorion is sufficient to support growth of the embryo. Initiation of cardiac contractions will continue aerobic metabolic processes to distribute oxygen to deep embryonic tissue.

Materials and methods

Animal care: Adult *P. pugio* were obtained from Gulf Specimen Marine Laboratories, Inc. (Panacea, FL, USA). Experimental animals were maintained in 40L aquaria in aerated artificial seawater (30-32 ppt at 20-25˚C) with a 12L: 12D photoperiod. Aquaria were maintained individually using filters with frequent water changes. Animals were fed a high protein marine fish flake to encourage ova production (Ocean Nutrition Formula Two Flakes). Conditions were maintained at 20° C, seawater 20 ppt, normoxic water (PO_2=20.5 kPa O_2) and 12L:12:D photoperiod.

Embryo collection: Ovigerous grass shrimp were attached to the flattened end of a wooden applicator stick on the lateral cephalothorax with cyanoacrylate glue. Embryos were removed from pleiopods of ovigerous females and transferred to nursery chambers

located on a continuously rotating platform to mimic movements of an ovigerous female. Conditions were maintained at 20° C, seawater 20 ppt, normoxic water (PO_2=20.5 kPa O_2) and 12L:12:D photoperiod.

Lactate assays: Embryo clutches (n = 7 – 11) were harvested and placed in 0.5 ml Eppendorf tubes with 0.1 ml of Tris(hydroxymethyl)aminomethane (Tris) buffer solution. The tubes were flash frozen with liquid nitrogen to prevent tissue and protein degradation and stored in a -80 degree Celsius freezer. Experimental assays were conducted according to the protocol described by the lactate assay kit provided by Sigma-Aldridge (MAK064 Sigma-Lactate Assay Kit, Sigma-Aldrich Co. LLC, United States). Minor changes were made to the protocol to accommodate for mass sample of *P. pugio* clutch and include: 1nmole/µL standard solution, lactate standard final concentrations ranging from 0 – 0.3 nm/µL and 50 µL of sample per well.

Statistical analysis: Means and standard deviations were calculated for each developmental stage (n = 3 – 6) for lactate accumulation data on embryos raised under normoxic conditions (18.5 kPa O_2 – 18.0 kPa O_2). The overall effects of development on the various dependent variables were studied using an analysis of variance (ANOVA) and Tukey *t*-test (PASW Statistics 18) and used to account for effects of oxygen availability. Multiple pairwise comparisons were conducted where a significance of $P \leq 0.05$ was accepted to reject the null hypothesis.

Results

Overall lactate concentrations from embryonic stages IIIb through VIII indicate a significant decreasing trend in lactate accumulation within the embryo when raised under normoxia (18.5 kPa O_2 – 18.0 kPa O_2, p = 0.001) (Figure 1). Lactate concentration significantly decreases upon initiation of cardiac contractions in stages VIb, VIIb and VIII (p = 0.001, n = 3 – 6).

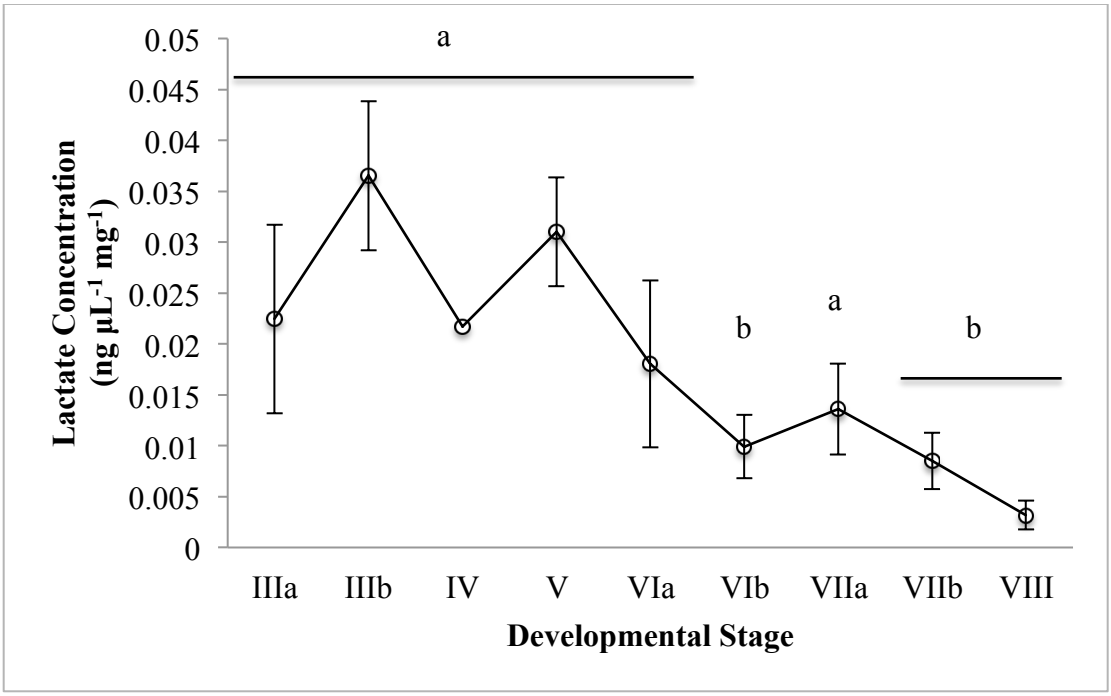

Figure 1. Lacate Accumulation During Development in Normoxia (18.5 kPa O_2 – 18.0 kPa O_2). Stages VIb, VIIb and VIII have significantly lower lactate accumulation compared to stage V (p = 0.001, n = 3 – 6, n = 1 clutch of 100 – 200 embryos).

Discussion

Lactate data was collected on embryos raised under normoxic oxygen conditions, flash frozen and homogenized according to the lactate assay protocol provided by Sigma-Aldridge (MAK064 Sigma-Lactate Assay Kit, Sigma-Aldrich Co. LLC, United States).

In recent years, various theories about anaerobic metabolism in aquatic embryos have become popular. One theory states that an embryo, without internal convective processes, utilize aerobic metabolism until the slight anaerobic metabolism initiates (Ehlinger and Tankersley 2003). Initiation of anaerobic metabolism then cues the embryo to hatch in order for aerobic metabolism to continue. A molecular theory has been established using the arthropod, *Artemia fransciscana,* and supports that HIF-1 proliferation or pyruvate dehydrogenase complex is downregulation is a method of compensatory response to oxygen stress (Hand 2011).

Our data show a significant decrease in lactate accumulation after initiation of cardiac contraction at stage VIb. Stage IIIb through VIa embryos accumulate trace amounts of lactate and continue with normal development until hatching approximately 3 days after cardiac contraction initiation, during stage VIII. These data support that in grass shrimp embryos, lactate accumulation does not act as a physiological cue for hatching. Instead, it is possible that HIF-1 upregulation occurs as a compensatory mechanism in order for embryonic development to continue normally. Another possibility for the significant decrease in lactate accumulation is the fact that cardiac contractions aid in circulation of ambient oxygen towards embryonic inner cell mass. In this case, lactate stores may be metabolized or excreted through the chorion, allowing for embryonic tissue development to continue and proceed towards hatching.

Further molecular studies must be conducted to quantify HIF-1 and pyruvate dehydrogenase complexes in order to confirm molecular compensatory mechanisms in *P.*

pugio embryos. Future studies may assess lactate accumulation under various hypoxic conditions to discover if any compensatory mechanisms take place in the grass shrimp embryo during exposure to oxygen stress. These may include serial exposure to low oxygen conditions, ranging from mild to severe hypoxia in order to bracket when—if any—switch from aerobic to anaerobic metabolism occurs during development.

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CHAPTER 4 HYPOXIC EXPOSURE EFFECTS ON THE EMBRYONIC GRASS SHRIMP, *Palaemonetes pugio*

Research conclusions

Prior to this study, no data have detailed embryonic oxygen consumption ($\dot{M}O_2$) in a closed, normoxic system. This research provides greater understanding of development and physiology in grass shrimp embryos. Contributions from *Palaemonetes pugio* research will provide the physiological element to integrative research, shed light on development in aquatic environments and determine effects of exposure to extreme environmental conditions.

The oxygen consumption data delves into the understudied research sector of aquatic embryonic development. Data obtained from these studies details metabolic processes that occur under normoxic and hypoxic conditions (18.5 kPa O_2 – 18.0 kPa O_2 and 11.0 kPa O_2 – 10.5 kPa O_2, respectively). No prior oxygen consumption analysis has been established with grass shrimp embryos. Therefore, our normoxic oxygen consumption data provides the first normoxic baseline of $\dot{M}O_2$ per embryonic stage. Embryos raised under hypoxic conditions have a significantly higher $\dot{M}O_2$ across embryonic stages. Embryonic stage IIIb suggests a developmental window where the embryo must activate compensatory mechanisms, or perish.

Each data point can be used to further divide individual components of oxygen regulation mechanisms. Oxygen consumption was further extrapolated to determine critical oxygen pressure (P_{crit}) per stage. Stages following cardiac initiation indicate a non-significant decreasing trend in P_{crit}, suggesting oxyregulation occurs in later embryonic stages. This may contribute to the mechanism which grass shrimp embryos are able to develop, hatch and survive at extreme hypoxic levels as low as 3.0 kPa O_2. The embryo can tolerate lower oxygen levels as cardiac development proceeds. Despite exposure to lower oxygen levels, no anaerobic metabolism occurs in embryos raised under normoxic conditions. There are however, lactate traces present throughout development, but *P. pugio* embryos do not undergo anaerobic metabolism during development in normoxic conditions (18.5 kPa O_2 – 18.0 kPa O_2). Lactate accumulation significantly decreases once cardiac contractions begin at stage VIb. During initiation of the heart, oxygen circulates internally and breaks apart the hypoxic oxygen gradient to allow sufficient oxygen circulation within the inner cell mass of the embryo.

The normoxic baselines for oxygen consumption ($\dot{M}O_2$), critical oxygen pressure (P_{crit}) and lactate concentration have been established for *Palaemonetes pugio* embryos in this study. These data implicate a physiological control mechanism that grass shrimp embryos undergo to survive external development and short maternal care periods (McMahon 2001). This warrants future research focused on *P. pugio* embryo exposure to experimental oxygen levels ranging from saturated hyperoxia, to severe hypoxia and anoxia. Future work may investigate: whether variable oxygen conditions delay or accelerate cardiac development in embryonic stages; determine if developmental and

physiological changes contribute to increased survivorship of *P. pugio* in estuarine systems and assess whether anaerobic metabolism occurs during exposure to long-term hypoxia or anoxia. When these data are compared with normoxic baseline data, a more thorough description of *Palaemonetes pugio* embryonic development will contribute to the aquatic embryology field of study.

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APPENDIX

Annotated python script

Customized by Doctoral Candidate, Christopher M. Hardy, University of Nevada, Las

Vegas.

This program takes raw data files from oxysense and calculates $\dot{M}O_2$ from user defined oxygen ranges.

Import packages to use.
```
import csv
import os
import math
```

User inputs the directory where oxysense raw data files are located.
```
dir = raw_input("Enter full directory where files are located: ex. /Users/dir/folder: ")
```

User inputs full file path to metadata file.
```
metaDataFile = raw_input("Enter full file path to metadata file: ex.
/Users/dir/folder/metadata.txt: ")
```

User inputs desired oxygen ranges to calculate $\dot{M}O_2$. Here the user can provide two ranges: 1 normoxic range and 1 hypoxic range.
```
valueNorHigh = input("Normoxic High Range: ")
valueNorLow = input("Normoxic Low Range: ")
valueHypHigh = input("Hypoxic High Range: ")
valueHypLow = input("Hypoxic Low Range: ")
```

This function calculates $\dot{M}O_2$.
```
def findOxyRate(mBarStart,mBarEnd,timeStart,timeEnd, massFactor, volumeFactor):
        timeDiff = timeEnd - timeStart
        mBarDiff = mBarStart - mBarEnd
        torrDiff = mBarDiff * 0.75006
        solubility = torrDiff * 1.4823
        molOxy = (solubility*(volumeFactor/1000))/(62.363*293)
        oxyRate = molOxy / massFactor / timeDiff
        print oxyRate
        return oxyRate
```

These lists are created to hold data that is calculated later in the program.

```
metaData = []
stageFactorList = []
skipList = []
stageNames=[]
normoxiaValues=[]
hypoxiaValues=[]

### Transfers contents of metadata file to metaData List ###
with open(metaDataFile, 'Ur') as x:
        metadata = csv.reader(x, delimiter='\t')
        for row in metadata:
                metaData.append(row)

### Iterates through the raw oxysense files and pulls out time, PO2 and corresponding
metadata information ###
for f in os.listdir(dir):
        fileName, fileExtension = os.path.splitext(f)
        if fileExtension == '.csv' and fileName != 'output':
                for row in metaData:
                        if f in row:
                                timeFactor = int(row[1])
                                stageFactor = row[2]
                                massFactor = float(row[3])
                                volumeFactor = float(row[4])
                                mBarList=[]
                                timeList =[]
                                with open(f, 'Ur') as r:
                                        file = csv.reader(r, delimiter=';')
                                        for row in file:
                                                if row[4] == 'mBAR' or row[4] == 'NaN':
                                                        pass
                                                else:
                                                        mBarList.append(row[(4)])
                                        for i in range(0, len(mBarList)):
                                                timeList.append(((i*timeFactor)/60.))

                                        zipList = zip(timeList,mBarList)
                                        finalTimeList = [float(row[0]) for row in zipList]
                                        finalmBarList = [float(row[1]) for row in zipList]
                                        timeListTrash = timeList
                                        timeList = []
                                        mBarListTrash = mBarList
                                        mBarList =[]
                                        finalList= zip(finalTimeList,finalmBarList)

                                        def findHighVal(pressure,finalList):
```

```
                import math
                idx = []
                for row in finalList:
                        if math.fabs(row[1]-pressure) <= 1:
                                idx.append(row[0])
                for x in idx:
                        if x == min(idx):
                                for row in finalList:
                                        if row[0] == x:
                                                HighMBar =
row[1]
                                                HighTime =
row[0]
                                                if HighMBar
is not None or HighTime is not None:
                                                        return
HighMBar, HighTime

        def findLowVal(pressure,finalList):
                import math
                idx = []
                for row in finalList:
                        if math.fabs(row[1]-pressure) <= 1:
                                idx.append(row[0])
                for x in idx:
                        if x == max(idx):
                                for row in finalList:
                                        if row[0] == x:
                                                LowMBar =
row[1]
                                                LowTime =
row[0]
                                                if LowMBar is
not None or LowTime is not None:
                                                        return
LowMBar, LowTime


        stageNames.append(f)
        stageFactorList.append(stageFactor)
        print f
        print massFactor
        print volumeFactor

        if findHighVal(valueNorHigh,finalList) is None:
                normoxiaValues.append("Out of Range")
```

```
                print "Normoxic Out of Range"
            elif findLowVal(valueNorLow,finalList) is None:
                normoxiaValues.append("Out of Range")
                print "Normoxic Out of Range"
            else:
                high = findHighVal(valueNorHigh,finalList)
                for a in range(0,len(high)):
                    b, c = high
                    mbarStart = b
                    timeStart = c
                low = findLowVal(valueNorLow,finalList)
                for x in range(0,len(low)):
                    y, z = low
                    mbarEnd = y
                    timeEnd = z
                print mbarStart, mbarEnd, timeStart,
timeEnd

        normoxiaValues.append((findOxyRate(mbarStart,mbarEnd,timeStart,timeEnd,ma
ssFactor,volumeFactor)))

            if findHighVal(valueHypHigh,finalList) is None:
                hypoxiaValues.append("Out of Range")
                print "Hypoxic Out of Range"
            elif findLowVal(valueHypLow,finalList) is None:
                hypoxiaValues.append("Out of Range")
                print "Hypoxic Out of Range"
            else:
                high =
findHighVal(valueHypHigh,finalList)
                for a in range(0,len(high)):
                    b, c = high
                    mbarStart = b
                    timeStart = c
                low = findLowVal(valueHypLow,finalList)
                for x in range(0,len(low)):
                    y, z = low
                    mbarEnd = y
                    timeEnd = z
                print mbarStart, mbarEnd, timeStart,
timeEnd

        hypoxiaValues.append((findOxyRate(mbarStart,mbarEnd,timeStart,timeEnd,mass
Factor,volumeFactor)))
```

```
### Calculates averages, standard deviations for each stage and oxygen range. ###

summary = zip(stageNames, normoxiaValues, hypoxiaValues, stageFactorList)

averageNames = []
blanks = []
nstageI = []
nstageII = []
nstageIIIa = []
nstageIIIb = []
nstageIV = []
nstageV = []
nstageVIa = []
nstageVIb = []
nstageVIIa = []
nstageVIIb = []
nstageVIII = []
hstageI = []
hstageII = []
hstageIIIa = []
hstageIIIb = []
hstageIV = []
hstageV = []
hstageVIa = []
hstageVIb = []
hstageVIIa = []
hstageVIIb = []
hstageVIII = []
for row in summary:
        if row[3] == "":
                blanks.append(0)
        elif row[3] == "I":
                nstageI.append(row[1])
                hstageI.append(row[2])
        elif row[3] == "II":
                nstageII.append(row[1])
                hstageII.append(row[2])
        elif row[3] == "IIIa":
                nstageIIIa.append(row[1])
                hstageIIIa.append(row[2])
        elif row[3] == "IIIb":
                nstageIIIb.append(row[1])
                hstageIIIb.append(row[2])
        elif row[3] == "IV":
                nstageIV.append(row[1])
                hstageIV.append(row[2])
```

```
elif row[3] == "V":
        nstageV.append(row[1])
        hstageV.append(row[2])
elif row[3] == "VIa":
        nstageVIa.append(row[1])
        hstageVIa.append(row[2])
elif row[3] == "VIb":
        nstageVIb.append(row[1])
        hstageVIb.append(row[2])
elif row[3] == "VIIa":
        nstageVIIa.append(row[1])
        hstageVIIa.append(row[2])
elif row[3] == "VIIb":
        nstageVIIb.append(row[1])
        hstageVIIb.append(row[2])
elif row[3] == "VIII":
        nstageVIII.append(row[1])
        hstageVIII.append(row[2])


def average(stageList):
        if "Out of Range" in stageList:
                stageList.remove("Out of Range")
        if len(stageList) is 0:
                return "None"
        else:
                average = sum(stageList)/len(stageList)
                return average

def stdError(stageList):
        if "Out of Range" in stageList:
                stageList.remove("Out of Range")
        if len(stageList) < 3:
                return ""
        else:
                avg = average(stageList)
                variance = map(lambda x: (x-avg)**2, stageList)
                avgVariance = average(variance)
                stdDev = math.sqrt(avgVariance)
                stdError = (stdDev/math.sqrt(len(stageList)))
                return stdError

naverages = []
nstdErrors = []
```

```
	naverageLists =
[blanks,nstageI,nstageII,nstageIIIa,nstageIIIb,nstageIV,nstageV,nstageVIa,nstageVIb,nst
ageVIIa,nstageVIIb,nstageVIII]
	for list in naverageLists:
		if len(list) > 0:
			naverages.append(average(list))
			nstdErrors.append(stdError(list))
		else:
			naverages.append("None")
			nstdErrors.append("None")

	haverages = []
	hstdErrors = []
	haverageLists =
[blanks,hstageI,hstageII,hstageIIIa,hstageIIIb,hstageIV,hstageV,hstageVIa,hstageVIb,hst
ageVIIa,hstageVIIb,hstageVIII]
	for list in haverageLists:
		if len(list) > 0:
			haverages.append(average(list))
			hstdErrors.append(stdError(list))
		else:
			haverages.append("None")
			hstdErrors.append("None")

	averageNames = ["No
Stage","I","II","IIIa","IIIb","IV","V","VIa","VIb","VIIa","VIIb","VIII"]

	printAverage = zip(averageNames,naverages,nstdErrors,haverages,hstdErrors)

### Outputs all data to file 'output.csv' ###
	with open("output.csv", "wb") as s:
		writer = csv.writer(s)
		writer.writerow(['Normoxic High', 'Normoxic Low', 'Hypoxic High',
'Hypoxic Low'])
		writer.writerow([valueNorHigh, valueNorLow, valueHypHigh,
valueHypLow])
		writer.writerow([""])
		writer.writerow(['Stage','Avg. Normoxic O2','Std Error','Avg. Hypoxic
O2','Std Error'])
		writer.writerows(printAverage)
		writer.writerow([""])
		writer.writerow(['Stage Name', 'Normoxic O2 Consumption
Rate','Hypoxic O2 Consumption Rate','Stage'])
		writer.writerows(summary)
```

Metadata file:

Metadata table must be a tab deliminated text file. Easiest way to make this is to make it in Microsoft excel and save it as a .txt file.

File Name	Time (sec)	Stage	Mass (mg)	Volume (mL)
Oxysense File 1	Time between readings	Developmental Stage	Mass of embryo	Volume of chamber

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