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Embryonic Development of the Grass Shrimp, Palaemonetes pugio, and the Influence of Salinity on Cardiac Physiology

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EMBRYONIC DEVELOPMENT OF THE GRASS SHRIMP, *PALAEMONETES PUGIO,* AND THE INFLUENCE OF SALINITY ON CARDIAC PHYSIOLOGY

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ABSTRACT

Embryonic Development of the Grass Shrimp, *Palaemonetes pugio*, and the Influence of Salinity on Cardiac Physiology

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The grass shrimp, *Palaemonetes pugio*, inhabit the brackish waters along the Atlantic coast of North America and are a key component of the estuarine ecosystem. These decapod shrimp can tolerate large fluctuations in environmental parameters including daily and seasonal fluctuations in salinity between 0 to 55 parts per thousand (seawater at 30-32 ppt). Any observed distribution patterns of adult *P. pugio* in relation to salinity may be accounted for by their relative ability to tolerate a range in salinity and their ability to maintain internal water volumes and cardiac performance in the earliest life stage, the embryonic period. This thesis describes embryonic development and cardiac physiology of *P. pugio* embryos at standard conditions (20˚ C, 30-32 ppt seawater) and under the influence of hypersalinity (20˚ C, 45 ppt seawater). By investigating developmental physiology, fundamental mechanisms of cardiac compensatory responses can be evaluated in face of osmotic stress induced by variable salinity.

A concise description and understanding of embryonic development is necessary to provide the foundation for the further investigation of physiological development. Here, we establish for the first time, a staging scheme for the embryonic period of development for the grass shrimp using an 8-stage sequence. Under constant conditions, (20˚ C, 30-32 ppt), embryos

were described by observable morphological changes using photo- and video-microscopy. The mean time for embryonic development from fertilization to eclosion was 13 days and mean clutch size was 190 (±5) embryos per female. The heart begins to contract at stage VIb. Using standardized nomenclature for decapod development to describe the ontogenetic sequences and cardiac maturation of *P. pugio*, patterns of development and cardiac physiology can be more accurately addressed.

The cardiovascular system is the earliest system to become functional during development and so it can be utilized to examine developmental strategies of basic physiological processes. Specifically, cardiac function should be linked to embryonic maturation through the establishment of internal convective processes to meet increasing metabolic demands. Cardiac physiological parameters (heart rate, stroke volume, and cardiac output) have been characterized here in embryos of the grass shrimp under standard conditions (20˚ C, 30-32 ppt). Furthermore, the relationship between the initiation of cardiac function and embryonic growth was examined by measuring dimensional growth of the egg, water content and metabolizing mass. It was found that the initiation of cardiac contraction occurs during a time when the embryo outgrows the diffusive capabilities of the chorion. The transition to internal convection is a likely attempt to sustain metabolic homeostasis for further development and growth of the embryo.

The effects of osmotically driven water movements between animal and environment are known to influence cardiovascular function in many species. However, the embryos of the grass shrimp are exposed to similar fluctuations in salinity as are the adults yet lack the ability to osmoregulate. In order to examine the ability of grass shrimp embryos to adjust cardiac parameters in response to osmotically driven water exchanges, cardiac parameters (heart rate, stroke volume, and cardiac output) were measured in embryos of *P. pugio* when transferred from control conditions (20˚ C, 30-32 ppt) to hypersaline conditions (20˚ C, 45 ppt). In higher salinity seawater, embryos showed a significant decrease in embryonic water content, egg volume, and

surface area that likely suggest a decrease in the volume of hemolymph within the cardiovascular system. In response, embryos showed observable compensations for hemolymph volume loss by increasing heart rate and decreasing stroke volume to maintain optimal levels of cardiac output that are necessary for convective transport. Demonstrations of a homeostatic role in cardiac responses to environmental stress may imply early development of cardiac regulation necessary for *P. pugio* survival.

Invertebrate cardiovascular physiology holds fundamental clues to evolutionary conserved mechanisms of cardiac regulation that exist in many advanced animal forms. Early developmental patterns in physiological ontogeny also supply a basic understanding of homeostatic function in adults. By investigating the effects of fluctuating salinities on cardiovascular development and physiology in the grass shrimp, *P. pugio*, we can better understand their environmental tolerances and survivability. This can lead to accurate predictions of the effects of future global climate change on decapod populations.

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CHAPTER 1

INTRODUCTION

THE GRASS SHRIMP, *Palaemonetes pugio*

Prawns of the genus *Palaemonetes* have a wide distribution across an array of aquatic habitats ranging from freshwater to brackish and saltwater ecosystems and are distributed throughout the world (Anderson, 1985; Knowlton and Kirby, 1984). The grass shrimp, *Palaemonetes pugio*, inhabit the brackish waters along the Atlantic and Gulf Coasts of North America (Anderson, 1985; Glas *et al*., 1997), and are highly abundant in estuarine systems which are characterized by variable environmental conditions. These decapod shrimp can tolerate large fluctuations in environmental parameters such as temperature, dissolved oxygen, and salinity (Anderson, 1985; Morgan, 1980; Roesljadi *et al*., 1976; Thorp and Hoss, 1975; Welsh, 1975; Wood, 1967). They are able to successfully survive and in many instances, reproduce in these environments that may range from salinities of between 0 to 55 parts per thousand (seawater at 30-32 parts per thousand; ppt), temperatures of 5˚ to 38˚C, and dissolved oxygen levels from 4 to 22 kPa (Anderson, 1985; Brown-Peterson *et al*., 2008; Cochran and Burnett, 1996; Knowlton and Kirby, 1984; Welsh, 1975). Since most physiological processes require a stable osmotic and ionic environment, salinity is generally considered a key ecological factor in coastal and estuarine zones (Anger, 2003). The direct effects of osmotic stress along with tangential influences have been previously reported and may select for physiological adaptations to such highly variable salinity environments (Anger, 2003; Lee and Petersen, 2003; McGaw and McMahon, 1996; Roesijadi *et al*., 1976; Roesljadi *et al*., 1976; Wheatly, 1988). Factors contributing to the seasonal and daily variability in salinity include rainfall, evaporation, and tidal movements (Anderson, 1985; Knowlton and Kirby, 1984; Welsh, 1975). This species' tolerance to

environmental fluctuations in salinity can be generally attributed to the ability of all decapods to regulate internal water volumes in relation to osmotic movements of water and ions (Mantel and Farmer, 1983). The physiological responses of adult and juvenile life stages of *P. pugio* to salinity changes have been extensively investigated (Knowlton and Kirby, 1984; Wheatly, 1988), but little is known about embryonic cardiac responses to changes in salinity.

The adults of *P. pugio* are euryhaline osmoregulators and therefore can maintain internal osmotic and chloride balance over a wide range of external salinities (Knowlton and Kirby, 1984; Roesljadi *et al*., 1976). The effects of salt and water balance between external media of decapods and their internal fluids follow the physical laws of thermodynamics characteristic of a two-compartment system separated by a semi-permeable membrane. Thus, strong differences in concentrations between the external medium and the animal's hemolymph results in diffusive movements of water and ions into and out of the animal across any permeable surfaces (Mantel and Farmer, 1983). When in dilute seawater, the animal takes on water leading to increased internal pressures and tissue swelling along with a loss of ions to the environment. Internal osmotic problems in freshwater crustaceans are primarily those of control of hemolymph volume, and cardiovascular compensation, as well as prevention of the loss of salts and the compensatory uptake of salts from the environment across the gill epithelium. Freshwater decapods also adjust by excreting a large volume of dilute urine from the antennal gland (Gilles and Pequeux, 1983; Mantel and Farmer, 1983; Wheatly, 1988). In concentrated saline environments, the animal risks becoming dehydrated and losing water to the environment across the tissues and exoskeleton and a diffusive gain of ions across the gill and gut epithelium. To retain water as much as possible, the animal excretes low volumes of concentrated urine (Gilles and Pequeux, 1983; Mantel and Farmer, 1983).

Palaemonetes pugio AS A MODEL SYSTEM

Certain characteristics of *P. pugio* allow their easy use as a model research organism for laboratory investigations. They are easily maintained in the laboratory and the length of their breeding season extends from February through October which allows for a sufficient supply of embryos most of the year. Females can produce large clutch sizes, ranging between 200 and 400 eggs, multiple times throughout the breeding season (Alon and Stancyk, 1982; Anderson, 1985; Welsh, 1975). The grass shrimp have a translucent exoskeleton, allowing many physiological parameters to be observed externally without the use of invasive techniques. In addition to their physical characteristics, these decapod crustaceans can provide insights into the evolution of the development of physiological and regulatory mechanisms. Palaemonids inhabit a variety of ecological niches and equally possess the ability to tolerate a range environmental stresses (Alon and Stancyk, 1982; Anderson, 1985). This species provides comparative and developmental physiologists a tractable model for furthering our understanding of developmental, physiological and environmental questions.

Characteristic of most Carideans, *P. pugio* embryos develop with eggs brooded externally in a translucent chorion on the pleopods of the females (Anderson, 1985; Bauer and Abdalla, 2000; Glas *et al*., 1997; Knowlton and Kirby, 1984). The eggs adhere to the pleopods by setae and the embryos are carried by the female, exposed to the ever changing environment. Normally, embryos hatch to become larvae 11-13 days post-oviposition under field conditions (Rayburn *et al*., 1996). While there are many studies regarding the harmful effects of environmental toxicity on development (Foss and Rayburn, 1997; Harper-Arabie *et al*., 2004; Rayburn *et al*., 1996; Tyler-Schroeder, 1979), there are few studies of natural embryonic and physiological development in this species.

ADULT CARDIOVASCULAR REGULATION IN RESPONSE TO SALINITY

The physiological responses of decapods to changes in environmental salinity have been extensively investigated (Anger, 2003; Knowlton and Kirby, 1984; McGaw and McMahon, 2003; Wheatly, 1988). Of these physiological changes, I am interested in the effect of salinity on cardiovascular parameters due to osmosis between the animal and the environment. Decapod crustaceans have what is described as in incompletely closed circulatory system (in contrast to the early characterized "open" system). Blood, termed hemolymph, is circulated within a complex arrangement of vessels and sinuses with oxygen taken up at the gills (McMahon *et al*., 1997; Reiber and McGaw, 2009). The term hemolymph is used because this fluid is a mixture of both blood and lymph (McMahon *et al*., 1997). As osmoregulators, these animals maintain a gradient between hemolymph osmotic pressure and that of their external medium and thus can experience osmotically driven movements of water across the body surface, gills and gut (Knowlton and Kirby, 1984). The result can be changes in internal hemolymph volume and resultant compensatory changes in cardiac performance may occur (McGaw and Reiber, 1998; Wheatly, 1988). Changes in cardiac function resulting from hemolymph volume changes have not been investigated during embryonic development.

The decapod crustacean's globular heart consists of a single muscular ventricle suspended within a pericardial sinus by suspensory ligaments. Upon muscular contraction, hemolymph is forced out of the heart, stretching the ligaments. Hemolymph flow is directed through multiple arterial outlets through the opening and closing of arterial valves located at the entrance of each vessel. The potential energy stored in the ligaments allows the heart to return to presystolic volume as hemolymph refills the heart via ostial valves in the ventricular wall (Guadagnoli *et al*., 2007; McMahon *et al*., 1997; Reiber and McGaw, 2009). Cardiac compensatory responses resulting from hemolymph volume changes can be observed and the dynamics of the system characterized. Heart rate and stroke volume of crustaceans are controlled

independently by nervous input from the cardiac ganglion and the central nervous system. After exiting the ventricle, hemolymph flows through a network of arterial systems and finally into capillary-size vessels that ramify into tissues for gas exchange. These vessels cannot be termed capillaries due to the absence of an endothelial lining (Reiber and McGaw, 2009). Hemolymph then collects in large sinuses and veins as it moves toward the paired infrabrachial sinuses which direct the hemolymph to the gills for oxygenation. Hemolymph moves through the branchio-cardiac veins for delivery to the pericardial sinus where, upon the next cardiac contraction, is drawn back through multiple pairs of ostia leading into the ventricle of the heart (Guadagnoli *et al*., 2007; Reiber and McGaw, 2009).

EMBRYONIC EXPOSURE TO FLUCTUATING SALINITY

In many crustaceans, the heart begins beating during embryonic development. To understand phenotypic characteristics and regulatory capabilities of cardiac function in adult *P. pugio*, one must first understand the patterns of cardiac development and the environment that shape its function. Environmental fluctuations in salinity during embryonic development can cause the embryo to experience osmotically driven water movements across the chorion. As the cardiovascular system matures in later stages of development, water movements may result in cardiac compensatory responses that facilitate proper hemolymph volumes and pressures (McMahon and Wilkens, 1983). The postulated baroreflex in decapods, changes in cardiac function in response to changes in hemolymph volume (Burggren *et al*., 1990) is synonymous to the vertebrate response and maybe explained by the Frank-Starling Law of the heart. Here, the length-tension relationship of cardiac muscle tissue regulates both cardiac stroke volume and systolic pressure. An increase in hemolymph volume will increase venous return to the heart which in turn would result in an expansion of the ventricle. The increase in ventricular pre-

loading volume (end diastole) will shift the length tension relationship of the myofibrils overlap, where a greater tension can be generated by the myocardium. This would increase the stroke volume through a decrease in end systolic volume. The overall effect produces an increase hemolymph pressure through an increase in cardiac contractility. The reverse effect was also reported with a reduction in hemolymph volume resulting in a decrease in contractility (Burggren *et al*., 1990; McMahon and Wilkens, 1983). Grass shrimp can both survive and reproduce in euryhaline environments due to their ability to osmotically regulate their hemolymph in response to an ever changing environment (Knowlton and Kirby, 1984). While osmotic mechanisms of these changes have been investigated with respect to fluctuations in salinity, the downstream cardiovascular responses have not.

One successful feature of this group is their ability to adapt to ecological stress. Crustaceans have evolved a variety of mechanisms to regulate cardiovascular function in response to an array of environmental stresses (McGaw and McMahon, 1996; McMahon, 2001; McMahon *et al*., 2002; Reiber *et al*., 1997). Estuarine and intertidal ecosystems are subject to salinity change due to the effects of tidal inundation and freshwater run-off from the land (Anger, 2003; Wheatly, 1988). The magnitude and rate of salinity fluctuation can range from gradual seasonal shifts to sudden diurnal shifts (Wheatly, 1988; Wood, 1967). These changes can depend on the topology of the land, the drainage system and the effects of tides and currents. Decapods should have evolved the ability to compensate for external salinity shifts via cardiac regulatory mechanisms that result in the appropriate maintenance of cardiac function during periods of challenge (McGaw and McMahon, 1996; Wheatly, 1988).

RESEARCH OBJECTIVES

This research is presented as three unique components all focused on embryonic response to salinity change. First, I establish an embryonic developmental staging scheme under controlled laboratory conditions of 20˚ C, 12 hour photoperiod, and a constant salinity of 30-32% to determine, in later experiments, if changes to the osmotic environment modify cardiac parameters during development. Second, in order to understand the timing of cardiac initiation, cardiovascular morphology and cardiac functions are described throughout development through direct observations (morphology), and monitoring heart rate and stroke volume (physiology), under control and experimental conditions. Third, I identify whether regulation of cardiovascular physiology as a function of salinity exists by determining acute versus chronic effects on cardiac function by transferring embryos from control conditions to an increased salinity. I hypothesize that the grass shrimp, *P. pugio*, exhibit cardiac development and physiology in coordination with osmotically driven water volume changes from external salinities. If cardiac parameters including heart rate and stroke volume are significantly different for embryos exposed to alternate salinities, it can be inferred that one reason for this species' distribution along stressful estuarine regions could be due to adaptive plasticity in cardiac function in response to hemolymph volume changes.

STAGING SCHEME

To further our understanding of physiological regulation during embryonic development of *P. pugio*, we must describe and establish the timing of embryonic development. A common set of terms as used with other closely related crustaceans will be used to facilitate comparative studies between related taxa as well as a means to review intraspecific variation. In order to image the embryos properly, eggs harvested from the pleopods of females will be maintained

under controlled conditions. Optimal embryonic developmental conditions are 20˚ C with a consistent 12-hour day and 12-hour light cycle and frequent water changes of artificial sweater at 30 ppt salinity (Glas *et al*., 1997; Tyler-Schroeder, 1978).

CARDIAC DEVELOPMENT

Cardiac function will be described and further quantified through video dimensional analysis of the heart and the use of equations to calculate ventricular volumes throughout the stages of embryonic development. Microscopic imaging will allow for geometric measurement of the heart for a 3-dimensional reconstruction at each developmental stage. When cardiac contraction initiates, a dorsal view of the heart will be measured at end diastole (EDV; maximal distension), end systole (ESV; minimal distension). The change in ventricular volume between EDV and ESV is the stroke volume (V_s) for one cardiac cycle (V_s = EDV – ESV). The volume of hemolymph pumped with each stroke (V_s) times the number of heart beats per minute (f_H) is an index of hemolymph volume expelled per unit time termed cardiac output (V_b ; $V_b = V_s \times f_H$). The values for EDV and ESV of the heart will be measured by a videomicroscope equipped with a CCD camera and the use of the computer software program ImageJ (National Institutes of Health, Bethesda, MD, USA).

PHYSIOLOGICAL REGULATION IN RESPONSE TO SALINITY CHANGES

Cardiovascular parameters (f_H, V_s, V_b) were measured in embryos using the same videomicroscope and dimensional analysis techniques as mentioned above and further determined for the shrimp when transferred to different levels of salinity. For this, newly fertilized embryos were harvested from ovigerous females and transferred between salinities using small scale

treatment tanks (100 ml reservoirs). Measurements of individuals came from multiple clutches, yet which were determined to be at the same stage of development. It should be assumed that different clutches are genetically similar and that due to external fertilization, cross-fertilizing is possible to occur often. Their genetic relatedness should prove that any statistical differences in heart parameters between clutches are due to environmental conditions and not those which are genetic.

I plan to compare embryonic morphology and cardiac physiology in embryos maintained under standard marine salinities (30-32 ppt) and transferred to hypersaline conditions (45 pp). I hypothesize that embryos will exhibit internal water volume changes in response to fluctuating salinity and at stages of cardiac activity, are capable of cardiac responses to maintain levels of function. Significant differences in cardiac activity, as quantified by f_H and V_s at high salinities could suggest an induced response from changes of hemolymph volume. To my knowledge, this is the first study of decapod circulation that indicates hemolymph volume may be regulated by a change in cardiac performance during embryonic development. In addition, this research may suggest the invertebrate circulatory system may not be a primitively designed and less efficient system than in vertebrates.

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CHAPTER 2

EMBRYONIC DEVELOPMENT AND CARDIAC MORPHOLOGY OF THE GRASS SHRIMP, *Palaemonetes pugio* (CRUSTACEA, DECAPODA): EMBRYONIC STAGING

ABSTRACT

Decapod crustaceans are known for their variation in developmental patterns and their successful adaptation to diverse environmental conditions. The grass shrimp, *Palaemonetes pugio*, inhabits the brackish waters off the Atlantic and Gulf coasts of North America and are a critically important component of the food chain in these ecosystems. A clear description and understanding of early embryonic developmental processes is necessary in order for this species to be used for future physiological, ecological, developmental and toxicological investigations. Here, we establish a uniform staging scheme for the embryonic period of development in grass shrimp using an 8-stage sequence. Under constant conditions, (20º C, 30-32 ppt sea water), mean clutch size was 190 (±5) embryos per female and mean time for embryonic development from fertilization to eclosion was 13 days. Morphological changes and staging were documented using photo-microscopy and video-microscopy. Also described are cardiac morphological changes associated with embryonic development. The morphometrics of both embryonic development and cardiac ontogeny are provided. The morphological features described for *P. pugio* throughout embryonic development and the standardized embryonic staging scheme can be used to compare developmental patterns and timing when investigating questions related to an array of biological disciplines making this species a stronger model system.

INTRODUCTION

The grass shrimp, *Palaemonetes pugio*, Holthius, 1949, is a decapod crustacean native to North American coastal estuaries and collectively are an important model organism for physiological, developmental and environmental investigations (Bauer and Abdalla, 2000; Cházaro-Olvera, 2009). Shrimp populations range from Nova Scotia to Veracruz, Mexico (Anderson, 1985) and are an important component to ecosystems (Welsh, 1975; Wood, 1967). Their habitats are typically characterized by extreme environmental variation which leads to physiological stress (Welsh, 1975). This species' success is related to being uniquely adapted to tolerate wide fluctuations in environmental conditions such as temperature, salinity and dissolved oxygen (Alon and Stancyk, 1982; Thorp and Hoss, 1975; Welsh, 1975).

The embryonic development of crustaceans in a broader sense has been extensively reviewed (Anderson, 1973; Gore, 1985; Hartnoll, 1982). Developmental patterns in Decapods are highly variable both among and within classes yet are also characterized by a number of unified patterns (Anderson, 1973; Gore, 1985). The general pattern of embryonic development seen in this order proceeds from large yolky eggs with spiral cleavage, through naupliar stages, and followed by the development of post-naupliar segments as a forwardly flexed caudal papilla (Anderson, 1973; Gore, 1985). Overlying this basic pattern of development is a number of morphological and physiological adaptations made by individual species allowing exploitation of particular environments (Anderson, 1973; Hartnoll, 1982). Comparing embryonic developmental patterns along with descriptive staging schemes (Nazari (2000), Müller *et al*., (2003), Müller *et al*., (2007), one can begin to see the group's ability to fill an array of ecological niches. Palaemonid embryonic and larval development is highly sensitive to environmental conditions and as such well-described developmental stages are needed when investigating physiological and morphological plasticity induced by varying developmental conditions (Ituarte *et al*., 2005; Rayburn *et al*., 1996).

Embryonic development of *P. pugio* is initiated by external fertilization of the eggs. The male deposits a spermatophore on the ventral thorax of a mature female, near the opening of the gonopores. As eggs are extruded from the oviduct, they pass across the spermatophore to be fertilized. The eggs are manipulated by the female and attached to the pleopods on the ventral surface of the abdomen where they are adhered by setae (Anderson, 1985; Bauer and Abdalla, 2000; Glas *et al*., 1997). Once fertilized, cleavage is initiated and follows a spiral pattern, resulting in the blastomeres being oriented at an angle to the principle axis of the embryo (Carlson, 2003). The outcome of cleavage within most groups is a blastoderm surrounding a unitary yolk mass. In crustaceans that develop from large, yolky eggs the nauplius stage is retained in an embryonized form, allowing a greater degree of developmental maturation to occur within the chorion prior to eclosion (Anderson, 1973; Gore, 1985).

In addition to determining whole animal morphological changes during development, specific anatomical changes of the heart are a useful indicator of transition between developmental stages. Cardiac performance may reflect the impact of environmental variables on the shrimp during development. The heart is one of the first physiological systems to become active and in crustaceans the first contraction can be seen during the embryonic period (Reiber, 1997; Spicer, 2001; Spicer and Morritt, 1996). The ventricle continues to undergo anatomical development even after the initiation of cardiac contraction occurs (Spicer and Morritt, 1996). It is important to understand the morphological changes associated with embryonic development at both a gross and systems level in order to make comparisons in developmental timing under control and experimental conditions for these animals.

Developmental-based studies in *P. pugio* have been commonly used in investigations focused on ecological, toxicological, environmental, and physiological processes (Glas *et al*., 1997; Rayburn and Fisher, 1997; Rayburn and Fisher, 1999). The larval development of the grass shrimp has been described previously by Broad (1957) yet the embryonic development has not been described nor has a consistent standard for developmental staging been established. The

objectives of this research are to document the morphological and physiological changes associated with embryonic development in *P. pugio* and to establish an embryonic staging scheme that can be used as a basis for developmental, comparative and toxicological investigations. The introduction of consistent terminology and control-based measurements to describe embryonic development is used to generate an embryonic staging scheme that is founded on previously described developmental patterns in this and other closely related species (Müller *et al*., 2004; Müller *et al*., 2007; Nazari *et al*., 2000). The embryonic staging scheme can be used in conjunction with previously described larval staging schemes to investigate plasticity of development in response to environmental change as well as to develop questions focused on the adaptive significance of developmental patterns and timing in this species.

MATERIALS AND METHODS

Animal Maintenance

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 water changes scheduled as necessary. Animals were fed a high protein marine fish flake to encourage ova production (Ocean Nutrition Formula Two Flakes).

Embryonic Culture

Embryonic cultures were established separately from the adult aquaria to assure consistency of environment and synchrony in developmental age. Embryos were removed at the funiculus from the pleopods of the ovigerous females then staged under a dissecting microscope. Embryos used to document development were placed into 100 mL nursery chambers (glass

containers) maintained at 20˚C, 30-32 ppt seawater, 12L: 12D photoperiod and kept on an agitating platform to mimic natural maternal movements.

Imaging and Microscopy

Live embryos were observed within their intact, translucent chorion for ontogenetic staging and cardiac description using a CCD camera (World Precision Instruments) attached to a stereo-microscope (Leica MZ12.5; McBain Instruments, Chatsworth, CA, USA). Whole egg morphological measurements were made using an ocular micrometer on a Zeiss light microscope at a magnification of 6X. Measurements of the ellipsoidal egg include the length from rostrum to abdominal flexure as the long axis, and the perpendicular width as the short axis. The dorso-ventral and lateral spanning short axes of the egg were not differentiated due to their equal distance in measurements. These long and short axes were further used to calculate whole embryonic volume (volume of ellipsoid = $4/3\pi \cdot l \cdot 2w$). The heart of the embryo is located in the posterior thorax and can also be modeled as an ellipsoid. Morphological changes of the ventricle were described with video recordings from a high speed camera (Phantom V5.1, Vision Research, Wayne, NJ, USA) for enhanced imaging and measured with ImageJ, an imaging analysis program (National Institutes of Health, Bethesda, MD, USA). Mean whole egg mass was approximated from pooled masses from groups of five eggs and dividing by five. Embryos were removed from nursery chambers with a pipette, placed onto a weighing boat, and blotted dry to remove external water before being weighed on a microbalance (0.0001 mg accuracy; Cahn 21 automatic electrobalance, Cahn Instruments Div., Ventron Corp., Cerritos, CA, USA).

RESULTS

Under constant laboratory conditions (20˚ C, 32 ppt, 12:12 day: light cycle), *P. pugio* females had an average clutch size of 190 (±5). Embryos took an average of 13 days to complete

development. Eggs from a single brood were synchronous in development, and reached eclosion within hours of each other.

Table 1: Embryonic developmental timeline of *Palaemonetes pugio*, Holthius, 1949, at 20˚ C and salinity of 30 ppt.

Stage	Description	Days post-fertilization
I	Post-fertilization, oviposition	0
II	Cleavage	1
IIIa	Gastrulation: Blastopore	2
IIIb	Gastrulation: V depression	3
IV	Early Nauplius	4
V	Mid-Nauplius	5
VIa	Late Nauplius: C shaped embryo	7
VIb	Late Nauplius: Cardiac Contractions	8
VIIa	Post-nauplius: Eye pigmentation	9
VIIb	Post-nauplius: Eye condensation	11
VIII	Pre-hatch Embryo	13

Table 2: Linear measurements (mm) of the major axes (n = 40) of the ellipsoidal embryonic egg of *Palaemonetes pugio*, Holthius, 1949, and corresponding egg masses (mg) of whole embryos (n = 8). From the linear measurements, whole egg volume (mm^3) is calculated (n = 40). Data are shown in mean values and standard error of the mean for each developmental stage.

Stage	Long Axis (mm)	Short axis (mm)	Egg mass (mg)	Egg volume (mm^3)
I	0.691 ± 0.007	0.513 ± 0.003	0.1058 ± 0.003	0.0951 ± 0.001
II	0.648 ± 0.004	0.475 ± 0.002	0.1054 ± 0.004	0.0767 ± 0.001
IIIa	0.671 ± 0.003	0.510 ± 0.002	0.1164 ± 0.003	0.0913 ± 0.000
IIIb	0.692 ± 0.003	0.514 ± 0.002	0.1229 ± 0.002	0.0957 ± 0.001
IV	0.681 ± 0.003	0.527 ± 0.002	0.139 ± 0.005	0.0988 ± 0.001
V	0.733 ± 0.004	0.540 ± 0.002	0.1479 ± 0.008	0.1118 ± 0.001
VIa	0.728 ± 0.003	0.590 ± 0.002	0.1864 ± 0.009	0.1324 ± 0.001
VIb	0.767 ± 0.003	0.575 ± 0.004	0.1678 ± 0.003	0.1328 ± 0.002
VIIa	0.799 ± 0.003	0.586 ± 0.002	0.1854 ± 0.006	0.1435 ± 0.001
VIIb	0.873 ± 0.002	0.603 ± 0.002	0.2169 ± 0.006	0.1664 ± 0.001
VIII	0.895 ± 0.003	0.632 ± 0.003	0.2409 ± 0.007	0.1874 ± 0.002

Embryonic development

Embryonic development of *P. pugio* is characterized by eight distinct stages and defined by significant morphological changes. Of these stages, III, VI, and VII can be further separated into early and late sub-stages. These distinct stages are numbered I-VIII with substages lettered; a and b (Fig. 1; a-k).

Stage I: Post Fertilization (0-5 hours)

The fertilized egg is an ellipsoid and is composed primarily of a pale yellow yolk mass (YM) surrounded by a transparent chorion (Ch). No embryonic structures or cellular divisions are observed (Fig. 1a). At this stage, the mean long axis of the egg is 0.691 mm (±0.007) and the mean short axis is 0.513 mm (±0.003; Table 2). The mean mass of an individual embryo is 0.1058 mg (±0.003) and mean volume at this stage is 0.0951 mm^3 (±0.001). Stage I is initiated at fertilization and lasts approximately five hours until the zygote initiates cellular division (Table 1).

Stage II: Cleavage (5-24 hours)

The single egg begins dividing with surface furrows visible in a spiral axes pattern, maintaining the yolk mass centralized (Fig. 2a). At each round of cellular division, blastomeres (Bl) remain of equal size but increase in number, each showing a prominent nuclei (Nu) and clearly outlined by their cleavage furrows (CF) (Fig. 1b). The mean length of the long axis of the egg is 0.648 mm (±0.004), mean length of the short axis is 0.475 (±0.002) and mean egg mass is 0.1054 mg (±0.044) and mean volume is 0.0767 mm^3 (±0.001; Table 2). Stage II lasts approximately 24 h post-fertilization and terminates at gastrulation (Table 1).

Stage IIIa: Gastrulation, blastopore formation (24-48 hours)

Blastulation continues forming an outer lining of blastodermal cells that surround the central yolk mass (Fig. 2b). On the ventral aspect of the egg, a thickened disk of cells develops at one pole. This is the germinal disk and represents the formation of the blastopore (Bp) as it begins to invaginate inward (Fig. 1c). The mean length of the long axis of the embryo at this stage is 0.671 mm (±0.003), mean short axis is 0.510 mm (±0.002), and mean egg mass is 0.1164 mg (±0.003) and mean volume is 0.0913 mm^3 (±0.000; Table 2). The blastopore is the early substage (a) of gastrulation beginning at 48 h post-fertilization and lasting until the formation of a V-shaped depression on the superior side of the embryo (Table 1).

Stage IIIb: Gastrulation, V-depression (48-72 hours)

The late substage (b) of gastrulation starts when blastomeric cells on the superior side of the growing blastopore begin to migrate forward and separate (Fig. 2c), changing the shape of the pore to a V-depression (Vd). The forward projecting extremities and fixed base of the V correspond to the future optic lobes and caudal papilla respectively (Fig. 1d). Embryos have a mean length of 0.692 mm (±0.003) on the long axis and 0.514 mm (±0.002) on the short axis of the egg; mean egg mass is 0.1229 mg (±0.002) and mean volume is 0.0957 mm^3 (±0.001; Table 2). The V-depression occurs at 72 h post-fertilization (Table 1) and is the terminating feature of gastrulation.

Stage IV: Early Nauplius (72-96 hours)

The V-depression changes into the early nauplius in stage IV, as embryonic structures begin to take form and are discernable (Fig. 2d). The paired optic lobes (OL) migrate anteriorly and become posteriorly bordered by three naupliar appendages: the antennules (An), antennae (At), and mandibles (Mb). The posterior extremity of the embryo is the caudal papilla (CP) and will develop into the future segmented abdomen. The stomodeum (St) can be seen in the center

of the translucent embryo against the surface of the remaining lightly colored yolk (Fig. 1e). The mean long axis of the egg is 0.681 mm (±0.003), mean short axis is 0.527 mm (±0.002), mean egg mass is 0.1390 mg (±0.005), and mean egg volume is 0.0988 mm^3 (±0.001)(Table 2). The stage of the early nauplius lasts for 24 h and ends 4 d post-fertilization (Table 1).

Stage V: Mid-Nauplius (96-144 hours)

The primary dimension of growth of the embryo at this stage is along the long axis, with the optic lobes extending anteriorly and differentiating into the cephalic region of the animal (Fig. 2e). Of the naupliar appendages (NA), the antennules and antennae have lengthened and are directed posterior-ventrally. The caudal papilla lengthens and folds over itself ventrally. Limb buds of the five post-naupliar appendages (PnA) can be seen laterally from the caudal papilla. Development proceeds from an anterior to posterior direction forming an embryonized nauplius with all remaining yolk on the dorsal aspect of egg (Fig. 1f). Mean lengths of the embryo are 0.733 mm (±0.004) on the long axis, 0.540 mm (±0.002), mean egg mass is 0.1479 mg (±0.008), and mean volume 0.1118 mm^3 (±0.001; Table 2). Stage V is initiated at the folding of the caudal papilla, lasts for 48 h at 5 d post-fertilization, and terminates when the caudal papilla has stretched the length of the long axis of the egg (Table 1).

Stage VIa: Late Nauplius, C shaped embryo (5-6 days)

The early substage (a) of the late nauplius is initiated when the embryo has grown to form a C-shaped body on the ventral surface of the egg (Fig. 2f). The caudal papilla has extended over itself posteriorly and is now segmented. The optic lobes have reached the opposite pole on the anterior end of the egg where they will continue to develop. Post-naupliar appendages are further developed than earlier (Fig. 1g). Mean embryo length of the long axis is 0.728 mm (±0.003), mean short axis is 0.590 mm (±0.002), mean egg mass is 0.1864 mg (±0.009), and mean volume is 0.1324 mm^3 (±0.001) (Table 2). Substage (a) of the late nauplius is characterized by the

development of the C-shaped embryo prior to cardiac function and occurs at 7 d post-fertilization (Table 1).

Stage VIb: Late Nauplius, Initiation of cardiac contractions (6-7 days)

Substage (b) of late naupliar development is marked by the initiation of cardiac contraction. The heart is located between the remains of yolk mass and the growing caudal papilla. The embryo has become completely encircled, filling the inside of the chorion. The caudal papilla resembles the future abdomen with a rudimentary telson developing ventrally just posterior to the optic lobes. The optic lobes have enlarged and have become more translucent resting bilaterally on the anterior pole of the egg (Fig. 1h). The long axis of the egg has expanded to a mean of 0.767 mm (±0.003), mean short axis is 0.575 mm (±0.004), and the mean egg mass at this stage is 0.1678 mg (±0.003) with a mean volume of 0.1328 mm^3 (±0.002)(Table 2). Substage (b) of the late nauplius starts at 8 d post-fertilization and is terminated at the appearance of eye pigmentation approximately 24 h later.

Stage VIIa: Post-nauplius, Eye-pigmentation (7-9 days)

The initiation of stage VIIa occurs with the development of darkly pigmented cells forming the future eye (Ey). The eye begins as a linear array of pigmented cells that form on the posterior edge of both optic lobes. The abdomen (Ab) appears fully compartmentalized from the rest of the body with the remainder of yolk in the cephalothorax, posterior to the optic lobes. All appendages are fully lengthened and overlap one another due to the tightly curled position of the post-nauplius (Fig. 2g). Telsonic spines can begin to be seen on the edge of the extending telson (Te) (Fig. 1i). The mean long axis of the egg is 0.799 mm (±0.003), mean short axis is 0.586 mm (±0.002), mean egg mass is 0.1854 mg (±0.006), and mean volume is 0.1435 mm^3 (±0.001)(Table 2). Linear eye formation begins in the post-nauplius embryo at 9 d post-fertilization and terminates when the pigmented eye becomes more round in shape (Table 1).

Stage VIIb: Post-nauplius, Eye condensation (9-11 days)

Late stage post-nauplius, substage (b), begins when pigmented cells condense, changing the eye into a more rounded appearance (Fig. 2h). There is movement within the thoracic region underneath a carapace (Ca) as the formed hepatopancreas becomes more active (Fig. 1j). The mean long axis of the egg is 0.873 mm (±0.002), the mean short axis is 0.603 mm (±0.002), mean egg mass at this time is 0.2169 mg (±0.006), and mean egg volume is 0.1664 mm^3 (±0.001)(Table 2). Substage (b) of the post-nauplius occurs at 11 d post-fertilization and terminates when the development of the compound eye has completed (Table 1).

Stage VIII: Pre-hatch Embryo (288-312 h)

Embryonic development is complete. The eyes are large pigmented circles with ommatidia evident (Fig. 2i). The appendages extend from the cephalothorax to overlap the telson. The carapace surrounds the formed hepatopancreas (Hp), which now has four lobules that can be seen actively contracting. Some animal movement can be seen and scaphagnathite movements begin arhythmically. The telson is clearly segmented with the last segment having telsonic spines. The edge of the telson overlaps the extending rostrum from the head (Fig. 1k). The mean long axis of the egg is 0.895 mm (±0.003), the mean short axis is 0.632 mm (±0.003), mean egg mass is 0.2409 mg (±0.007), and the mean volume is 0.1874 mm^3 (±0.002) (Table 2). The embryo has fully developed at this stage (13 d post-fertilization) with eclosion occurring within hours (Table 1).

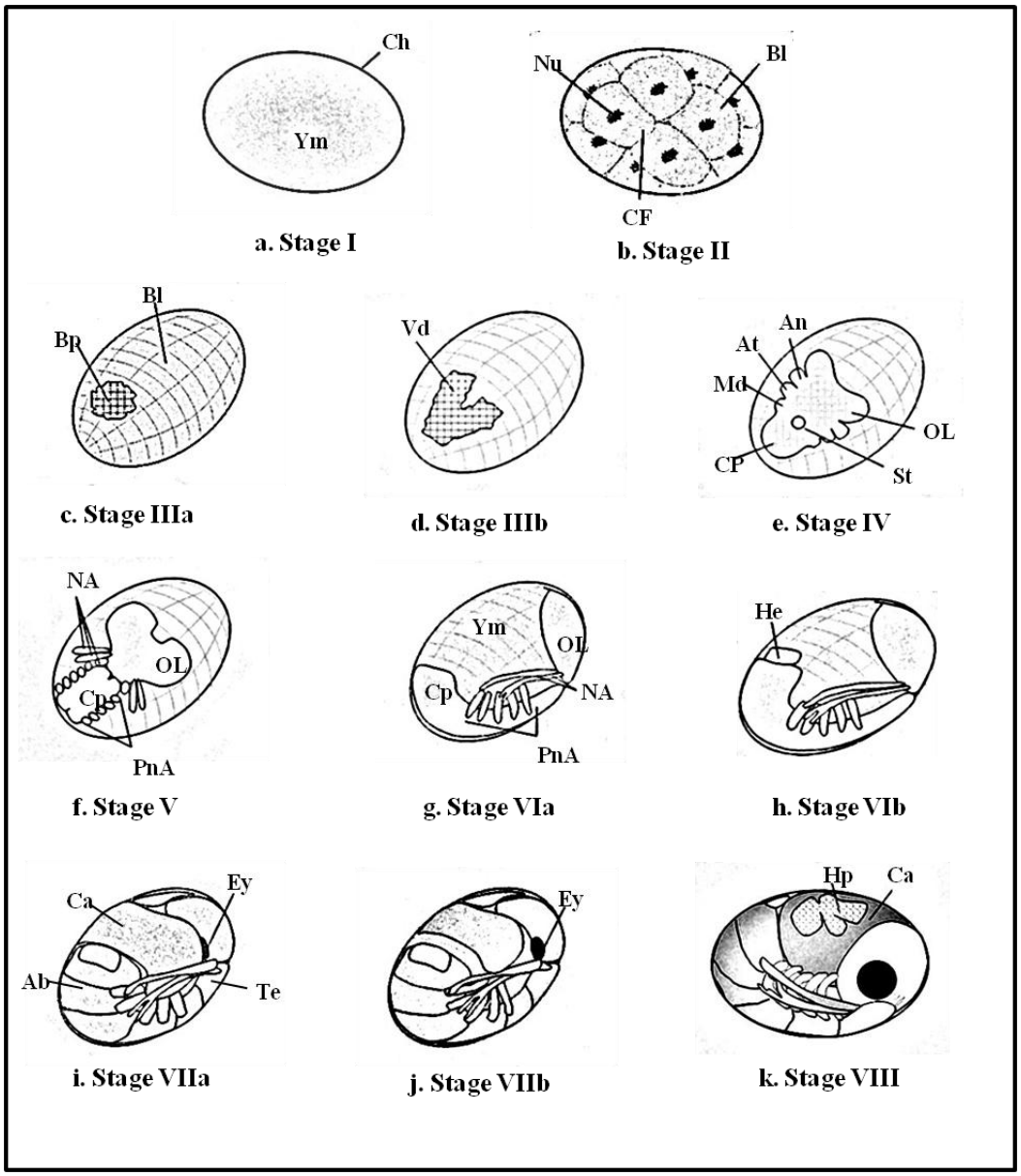

Figure 1: External characteristics of the embryonic developmental stages for *Palaemonetes pugio*, Holthius, 1949: (a.) stage I, fertilized egg; (b.) stage II, cleavage; (c.) stage IIIa, gastrulation with blastopore; (d.) stage IIIb, gastrulation with V-depression; (e.) stage IV, early nauplius; (f.) stage V, mid-nauplius; (g.) stage VIa, late nauplius with posterior segmentation; (h.) stage VIb, late nauplius with cardiac initiation; (i.) stage VIIa, post-nauplius with eye pigmentation, (j.) stage VIIb, post-nauplius with eye condensation; (k.) stage VIII, pre-hatch embryo. (Ab) Abdomen, (An) antennulae, (At) antennae, (Bl) blastomere, (Bp) blastopore, (Ca) carapace, (CF) cleavage furrow, (Ch) chorion, (CP) caudal papilla, (Ey) eye, (He) heart, (Hp) hepatopancreas, (Mb) mandibles, (NA) naupliar appendages, (Nu) nucleus, (OL) optic lobe, (PnA) post-naupliar appendages, (St) stomodeum, (Te) telson, (Vd) V-depression, (Ym) yolk mass.

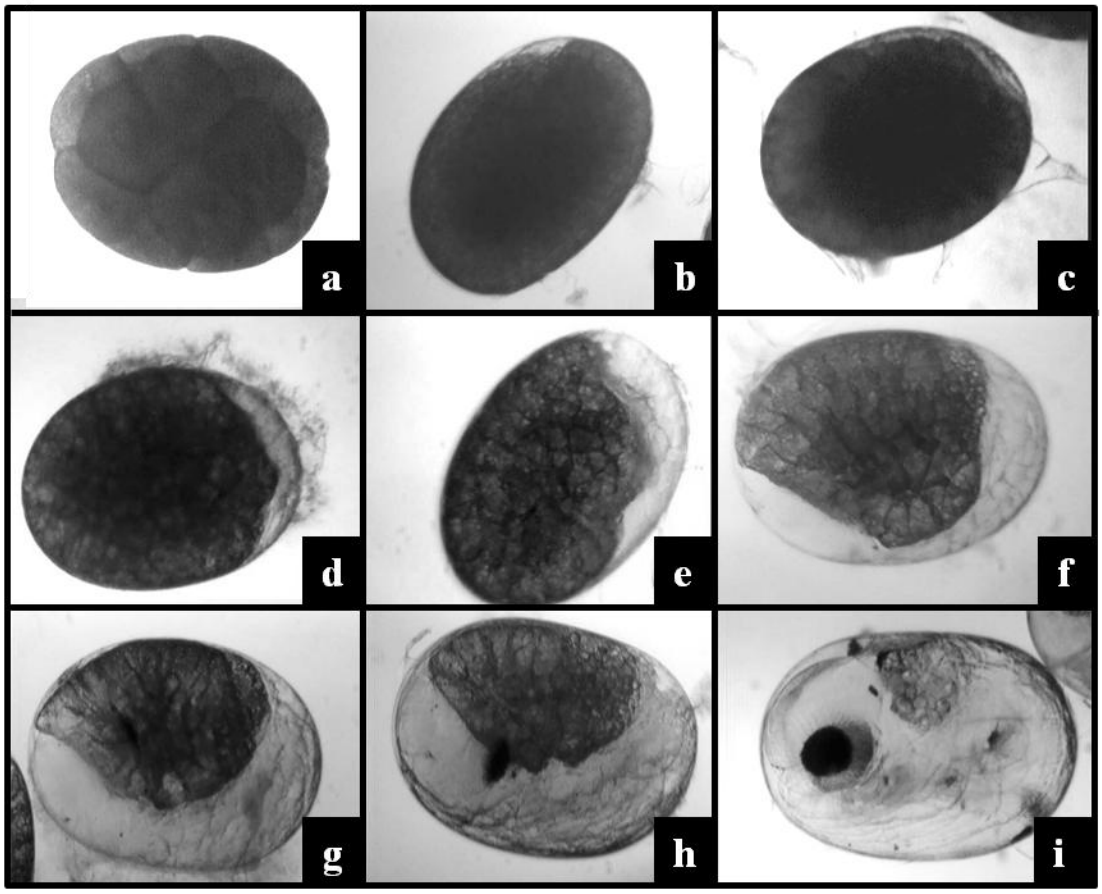

Figure 2: Images of embryonic stages of development of *Palaemonetes pugio*, Holthius, 1949, taken under a stereoscope. Stage I and stage VIb are omitted for irrelevance of single shot image properly representing characteristics for those particular stages. Stages pictured include: (a.) stage II, cleavage; (b.) stage IIIa, gastrulation with blastopore; (c.) stage IIIb, gastrulation with V-depression; (d.) stage IV, early nauplius; (e.) stage V, mid-nauplius; (f.) stage VIa, late nauplius with posterior segmentation; (g.) stage VIIa; post-nauplius with eye pigmentation; (h.) stage VIIb; post-nauplius with eye condensation; (i.) stage VIII; pre-hatch embryo. Images of animals shown here are not processed together to scale.

Cardiac Development

The heart of the embryonic grass shrimp, *P. pugio*, is a single muscular ventricle located in posterior end of the thorax (Fig. 3b; 4a-d). The formation of the inactive heart can be observed in the intact C-shaped embryo of stage VIa as a translucent structure out-pocketed from the

dorsally extending cardiac tube. Details of ostial and vasculature development cannot be seen until initiation of contraction occurs. The heart begins to beat at stage VIb of embryonic development. Contractions are arrhythmic and lack coordination between the lateral walls of the ventricle. At this time, the heart appears to be a laterally inflated region of the dorsal aorta which extends along the embryo both anteriorly and posteriorly (Fig. 3b). Vasculature supplying the anterior of the embryo beyond the aorta may exist but the primary pathway utilized is the single anteriorly extending aorta. It appears that valve control is weak in the anterior aorta having observed masses suspended within the hemolymph (most likely particles from yolk breakdown) exit incompletely through this route during contraction and returning back through upon relaxation of the ventricle. The posterior aorta runs superficially on the abdominal segment and, like the anterior aorta, can be seen to lack tight control of valvular function as re-entry of suspended masses within the hemolymph occur often upon relaxation of the heart. At this stage, a pair of bilateral ostia can be seen from the dorsal surface of the heart. Coordination of ostial closure is more organized at this stage, most likely because they act in affect of stronger contractions not independently regulated. And yet, there are masses suspended within the hemolymph that travel back and forth through the ostia during contraction and relaxation as seen in the aortic vessels. Additional ostial pairs may exist and either cannot be seen because of a possible ventral location that is obscured by the animal folding and/or because they do not open or close during contraction and relaxation. Visualization of these structures in live embryos depends heavily upon their differential movement during the cardiac cycle.

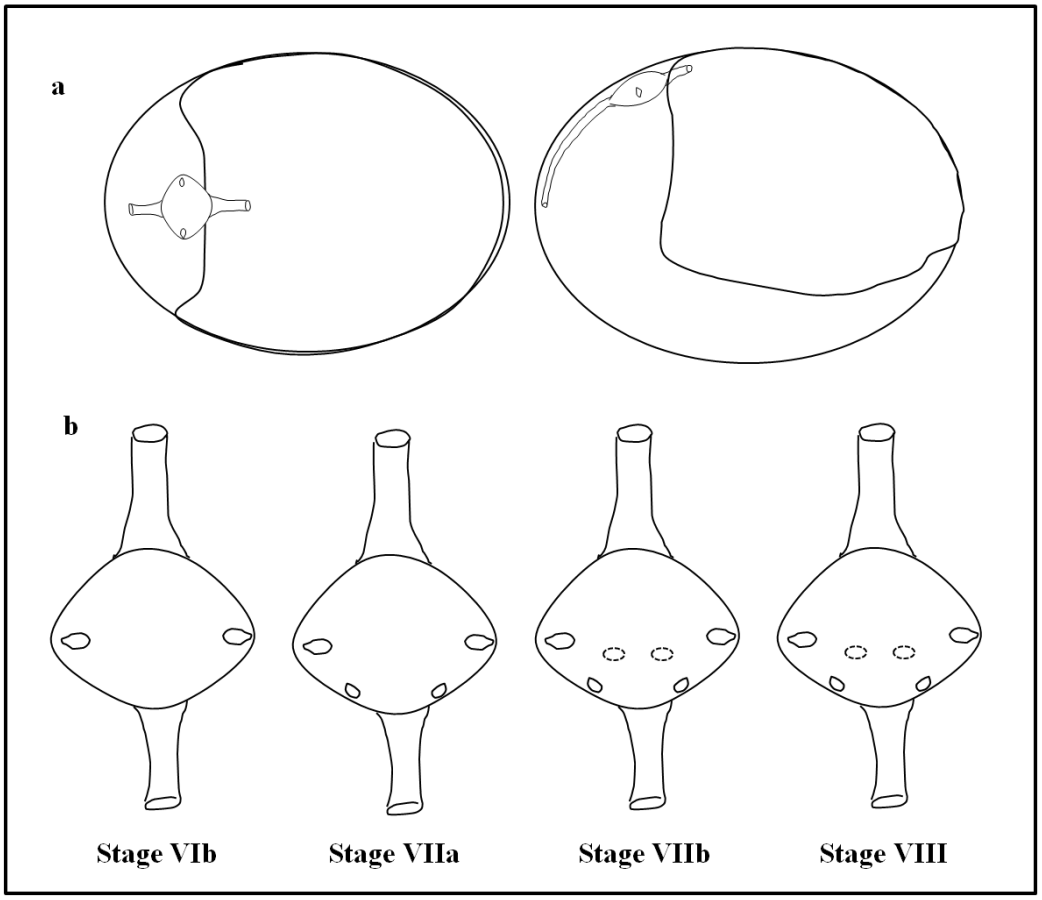

Figure 3: Illustrations of the dorsal view of the ventricle during embryonic stages of *Palaemonetes pugio*, Holthius, 1949: Dimensional arrangement of the ventricle for analysis; the Y-axis runs the length of the long axis of the egg, (a.) Dorsal and lateral positions of the heart in reference to the embryonic shrimp, (b.) Morphological development of the ventricle during stages of embryonic development. Functional ostial pairs can be seen developing through the stages. Solid lines signify a structure seen on the dorsal surface of the heart and dashed lines signify a structure seen through the heart on the ventral surface.

In stage VIIa, contraction has become more regular and is synchronous between the lateral edges of the ventricle (Fig. 3c). In addition to the dorsally located pair of ostia seen in the previous stage, a new pair of ostia can be seen operating posteriorly on the lateral edges of the ventricle. These ostia appear shorter and function over a smaller area on the surface of the ventricle.

When embryonic development has reached stage VIIb, cardiac contractions continue to become more organized and ostial function easier to discern (Fig. 3c). A functional third pair of ostia on the ventral surface can be seen through the translucent ventricle.

At stage VIII of development, the heart is coordinated and contraction is rhythmic with the ventricle expanding to the boundary of the pericardial sac (Fig. 3c). While no notable changes can be seen to have occurred between stages VIIb and VIII of development, structures surrounding the heart are more defined such as the hepatopancreas. During contraction, a definite pulling can be seen on the pericardial sac even though no suspensory ligaments can be seen to connect to the ventricle.

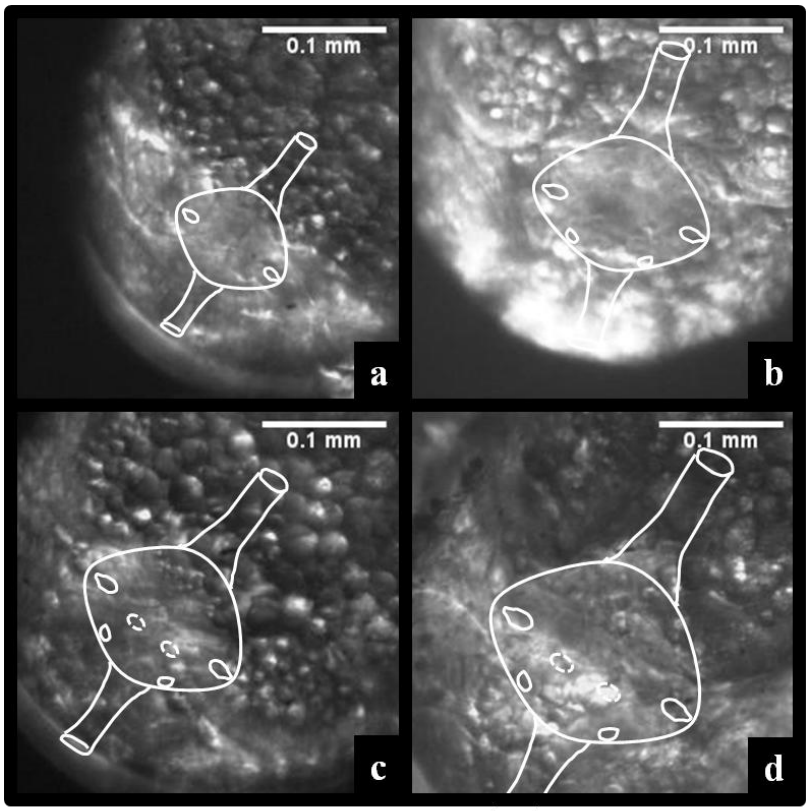

Figure 4: Images of the dorsally positioned ventricle in embryonic stages of development in *Palaemonetes pugio*, Holthius, 1949: (a.) stage VIb, (b.) stage VIIa, (c.) stage VIIb, (d.) stage VIII. The ventricle is seen outlined as an ellipsoid. Images shown are pictured to individual scale bars.

DISCUSSION

Embryonic development of *P. pugio*

In establishing a developmental staging scheme, it is important to have consensus with other ontogenetic patterns outlined in previous studies produced for closely related species. While staging nomenclature varies somewhat among related species, comparable descriptive analyses of embryonic development exist (for *Macrobrachium acanthurus*, see Müller *et al*., 2007, and *Palaemonetes argentinus*, see Nazari *et al*., 2000) and were used to establish an embryonic staging scheme for *P. pugio*. A comparative characterization of embryonic development was made for *Macrobrachium olfersi*, *Macrobrachium potiuna*, *Palamon pandaliformis* and *Palaemonetes argentinus* (Müller *et al*., 2004). Anger *et al*., (2002) made a comparative analysis of egg composition and size in species of Palaemonidae, Atyidae, and Pandalidae. Additionally, embryonic developmental patterns outlined in other studies using *P. pugio* were reviewed and key developmental landmarks were incorporated to establish a more consistent staging scheme (Tyler-Schroeder, 1978; Tyler-Schroeder, 1979). There are many similarities in decapod development that are made evident in these studies including the morphological progression of development from dividing cells through organogenesis to an embryonized animal form termed the nauplius. However, there are species specific differences such as developmental rates and egg size. The development of the grass shrimp can be morphologically described in reference to existing studies along with direct comparisons of the timing of progression of crucial embryonic events.

Embryonic development of *P. pugio* begins with external fertilization, stage I, as the female deposits the eggs onto her pleopods. The eggs are centrolecithal showing a pattern of modified spiral cleavage, like that of most decapod crustaceans (Anderson, 1973; Gore, 1985). Subsequent divisions of blastomeres at stage II show clearly defined cleavage furrows yet, like that found in Müller *et al*.,(2004), dissection and removal of the chorion showed what has been

described as a holoblastic cleavage pattern with blastomeres incompletely divided from the yolk mass.

In stage III, once differentiation of blastomeres at one pole of the egg has formed the germinal disc, a thickening followed by an invagination occurs contributing to the blastopore and later, the V-depression (Müller *et al*., 2003; Nazari *et al*., 2000). The cells that migrate through the blastoporal area will constitute the early germ layers and it is at this stage in most Palaemonids that an intense organization of the embryonized nauplius begins (Hartnoll, 1982; Müller *et al*., 2004). This terminates the stages of development that contribute to pre-naupliar development; the period of cleavage and gastrulation. Pre-naupliar development has been shown to vary moderately in lengths of time for related Palaemonid species with *P. argentinus* lasting approximately 1 day and *P. pandaliformis* lasting between 2 and 3 days (Müller *et al*., 2004). We report here that *P. pugio* pre-naupliar development lasts for 3 days (Table 1).

The formation and duration of the embryonized nauplius begins at stage IV as the early nauplius and lasts until stage V, the mid-nauplius. Naupliar development has little variation in time among palaemonid species with *P. argentinus* and *P. pandaliformis* both lasting approximately 1 day (Müller *et al*., 2004). Our results follow this trend with the embryonized nauplius in *P. pugio* lasting 1 day (Table 1). The mid-nauplius, stage V, proceeds with the addition of abdominal segments initiating the group of stages contributing to post-naupliar development, stages VIa-VIII. Yolk reserves decrease as the lipid and protein content are metabolized into embryonic structures and the embryo develops from a simpler form, to a more complex post nauplius, stage VI. At the termination of this stage, the embryonic heart begins contracting, suggesting the need to initiate internal convective processes and the advent of cardiac regulatory capabilities.

Duration of post-naupliar development last until eclosion and varies most among palaemonid species compared to the pre-naupliar and naupliar stages. Post-naupliar development was reported to last between 10 and 21 days in related species by Müller *et al*., (2004) and lasted

7 days here for *P. pugio* until eclosion (Table 1). Stages VII and VIII show an increase in motor activity of post-naupliar appendages along with coordination of scaphagnathite beating and active vitellophage function of the rudimentary hepatopancreas. After embryonic development is complete, the embryo reaches eclosion and hatches out into the zoeal form.

There is a positive linear growth of the eggs during this period of development (Fig. 5a). Growth of the long axis (X axis) increased with a slope of 0.023 while growth of the short axis (Z axis) increased at a slope of 0.014. Volume increases similarly, growing at a slope of 0.065 during development (Fig. 5b). Egg sizes and development times are consistent with a common trend that appears in decapod crustaceans where smaller eggs have a shorter embryonic period than that off larger eggs. This has been described by Müller *et al*., (2004) where small eggs of in *M. olfersi* and *P. argentinus* have duration of development lasts 14 days and 12 days respectively. Yet with larger eggs, duration of embryonic development in *M. potiuna* was 21 days. Our data further suggest the importance of egg size on duration of development, where eggs with a larger yolk supply have the ability to retain developmental processes during the embryonic period before hatching out. The adaptive significance of a longer embryonic period should supply the developing embryo with nutrients and time to develop more complex structures and better fit for environmental conditions.

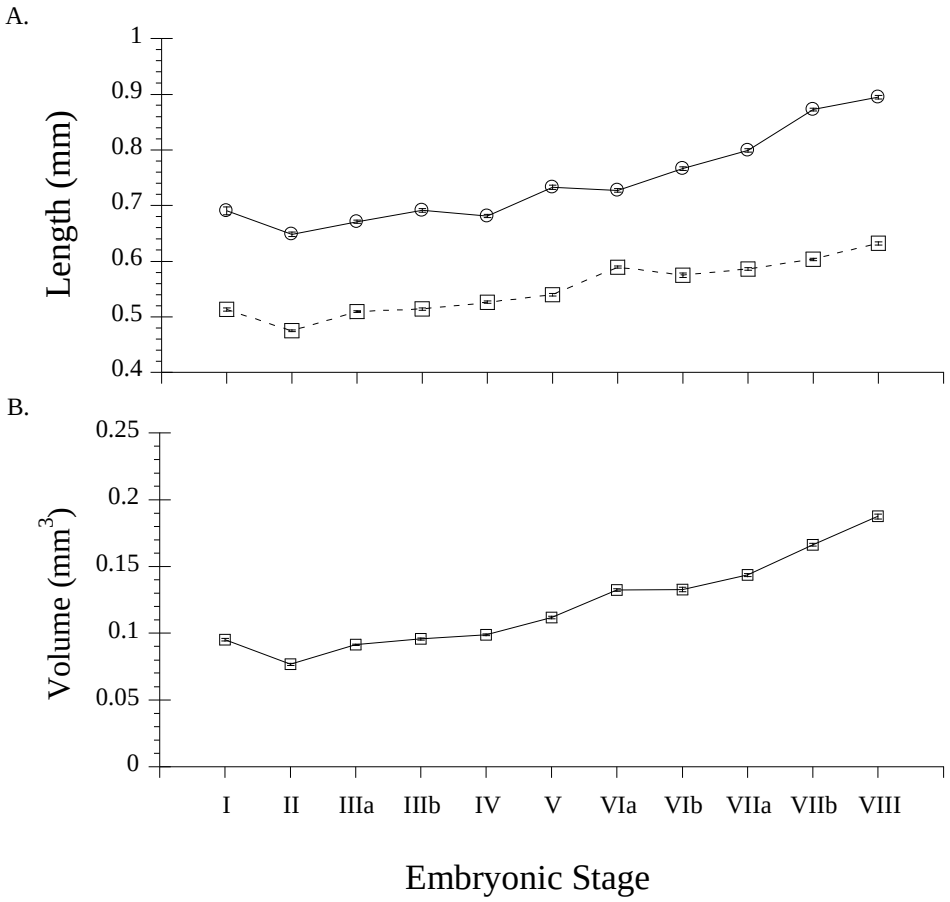

Figure 5: Linear measurements and volume of *Palaemonetes pugio*, Holthius, 1949, embryos: (a.) Linear growth over time in development with circles and squares representing the long and short axes respectively, and (b.) volume over time in development in calculated from linear measurements. Error bars are the standard error of the mean.

Cardiac development of *P. pugio*

Cardiac development of *P. pugio* was consistent with that seen in the adult grass shrimp (Guadagnoli *et al*., 2007). In stage VIb of embryonic development, when cardiac contractions are initiated, the bilaterally located ostial valve pair can be equated to the adult dorsal ostial valves. The heart is more rounded in the embryo than the adult thus using an ellipsoid rather than a trapezoid best describes the ventricle.

The second pair of functional ostial valves seen in stage VIIa of embryonic development can be equated to the lateral ostial valves described in the adult (Guadagnoli *et al*., 2007). This

set of ostial valves most likely did not form during this stage of development as ostial formation is inscribed at each segment of the dorsal heart tube in development (Wilkens, 1999), but rather began to function at this time. The same is seen for the third pair of ostial valves. Seen to function first in stage VIIb, these valves correspond to the ventral ostial valves described in the adult arrangement (Guadagnoli *et al*., 2007).

At stage VIII the heart has developed more tapered lateral edges and now resemble a trapezoidal as seen in the adult but due to the curvature of the animal within the egg, the heart is still best modeled as an ellipsoid. During development, primary vasculature includes the dorsal aorta that extends anteriorly and posteriorly from the ventricle. These are best described as the anterior aorta and posterior aorta in conjunction with terminology of the adult (Guadagnoli *et al*., 2007). Secondary vasculature is not detected, not necessarily because of the lack of formation but likely rather the lack of utilization during the embryonic period of the animal. At stage VIII, the anterior aspect of the ventricle appeared heavily rooted into the newly formed hepatopancreas suggesting existence of secondary vasculature. Further investigations using alternative imaging techniques may elucidate the details of these vessels.

A more comprehensive embryonic staging scheme for the grass shrimp along with a description of cardiac development is crucial for ecological, environmental, toxicological and physiological investigations using this important species. The literature is filled with studies using Palaemonid shrimp as models for an array of investigations where embryonic development is characterized by loosely defined terms or simple egg dimensions under a variety of laboratory conditions. Without a standardized embryonic developmental staging scheme, ontogenetically based events may be overseen, inaccurately characterized or even over stated. This description of development and corresponding cardiac maturation in embryonic *P. pugio* is crucial for studies using similar crustaceans. Whether used for studies of environmental interactions on morphology and developmental processes or broad scale evolutionary patterns of development, a standardized staging scheme based on define characteristics is necessary for a basis of comparison.

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CHAPTER 3

ONTOGENY OF CARDIAC FUNCTION IN THE GRASS SHRIMP *Palaemonetes pugio*

ABSTRACT

The cardiovascular system is the earliest system to become functional during development in an organism. As an animal continues to develop, the anatomy and physiology of the heart must also grow and mature to maintain proper physiological function. The ontogeny of cardiac physiology was investigated in the embryonic stages of the grass shrimp, *Palaemonetes pugio*, with specific regard to functional and anatomical development of the heart and growth of the embryo (dimensional growth, water content and metabolizing mass). The grass shrimp heart begins to contract at embryonic stage VIb, when incubated at 25˚C and 30-32 ppt salinity. The initiation of cardiac activity occurs just after an observed plateau in embryonic growth in terms of mass, surface area, egg volume and surface area-to-volume ratio. Once the heart begins to beat, these growth parameters resume their previous trajectories, which are followed by an increase in cardiac stroke volume (V_s) and heart rate (f_H). The product of both cardiac variables (f_H and V_s) results in an increase in cardiac output (V_b) as embryonic development continues. It is hypothesized that during embryonic development, initiation of contraction occurs when the embryo outgrows the diffusive capabilities of chorion, driving the need for internal convective processes to maintain metabolic homeostasis and allowing the embryo to continue to grow. Furthermore, the independent modulation of f_H and V_s with embryonic development is of interest as it appears these two cardiac parameters mature differentially.

INTRODUCTION

The grass shrimp is a model organism for the investigation of physiological responses to environmental change for a variety of reasons but of increasing significance is this organism's evolutionary history and ability to survive and thrive in highly variable environments including estuarine ecosystems. The relationship between organismal adaptation and its environment can be studied by examining the ontogeny of physiological processes in early life stages as this time period can be most sensitive to environmental disturbance and thus selection. The cardiovascular system is one of the first physiological systems to become functional during embryonic development and continues to mature and develop as the animal is exposed to continued ontogenetic change and environmental demands. Crustacean morphological developmental patterns are highly variable and so are their cardiovascular developmental strategies, which may explain not only the ability of this taxon to exploit a wide range of niches but also meet the challenges of variable and stressful environments (McMahon *et al*., 1997; Spicer, 2001; Wilkens, 1999).

Adult and larval crustacean cardiovascular development has been investigated with a focus on furthering our understanding of regulatory patterns and environmental influences (McMahon *et al*., 1997; Reiber and Harper, 2001; Spicer, 2001; Spicer and Morritt, 1996). However, it is apparent that the effect of stresses imposed during embryonic development are highly significant and must be assessed to gain an understanding of how the cardiovascular system of this important species responds, compensates and adapts. Grass shrimp are a key component to estuarine ecosystems and have been show to tolerate large temperature fluctuations, a range of oxygen concentrations and salinity variations (Welsh 1975). The ecological significance of this species has been extensively examined as has the impact of environmental toxins on this cornerstone of the food web (Harper-Arabie 2004). The ontogenetic sequence of cardiac function should be linked to embryonic growth through the establishment of internal

convective processes to meet increasing metabolic demands. The onset and timing of cardiac functions will be correlated with growth parameters to test this hypothesis. Additionally, based on our understanding of the importance of the embryonic period of development and the significance of this key indicator species, an investigation into the cardiovascular physiology, growth and development will establish an important base-line on which future studies can be launched.

In the range of diverse developmental patterns within decapods, *P. pugio* are described as exhibiting regular development. This is a relative term to describe the ontogeny of the individual group when compared to other closely related decapods, i.e. genera or family. Regular development is defined as the numerically predominant type of ontogenetic growth and developmental staging that consistently recurs in a particular decapod group" (Broad, 1957; Rabalais and Gore, 1985). This process can be compared to those patterns seen in what is termed "irregular development" (including a continuum between abbreviated and direct development to opposing extended development) (Gore, 1985; Rabalais and Gore, 1985; Reiber and Harper, 2001). Regular development of *P pugio* includes an embryonic developmental period which upon eclosion, is followed by a postnaupliar form, the freely swimming zoea. An embryonized nauplius implies that the development of many physiological processes occur prior to eclosion and full exposure to environmental variability. A longer embryonic period that is followed by multiple anamorphic larval stages can be advantageous to the survival of the animal because the embryo which resides within the chorion maybe buffered from environmental changes (Reiber and Harper, 2001).

Much of our understanding of crustacean cardiovascular function during development is based on larval studies and the systems treated as adults scaled down to size in terms of physiology, growth and compensatory responses (Spicer and Morritt, 1996; Wilkens, 1999). In many crustacean studies, the initiation of cardiac function occurs during the embryonic period

prior to eclosion (Harper and Reiber, 2004; Reiber, 1997; Spicer, 2001; Spicer and Morritt, 1996). It is believed that the initiation of heart function is linked to the need to facilitate internal convective process as simple diffusion across the chorion can no longer maintain an appropriate level of gas and nutrient exchange required to meet the embryos demands (Harper and Reiber, 2004; McMahon, 2001; McMahon *et al*., 1997; Reiber, 1997; Reiber and Harper, 2001; Spicer and Morritt, 1996). In this study we test the hypothesis that the onset of internal convective processes (cardiac contractions) occur at a point in development when growth plateaus as the embryo can no longer meet oxygen demands through simple diffusion across the chorion and that this key event then correlates with continued growth. Additionally, fundamental cardiac parameters will be assessed over the embryonic period (such as heart rate, f_H; stroke volume, V_s; and cardiac output, V_b) to determine how internal convective processes meet metabolic demands and if they appear to be regulated.

METHODS AND MATERIALS

Shrimp Culture

Grass shrimp, *Palaemonetes pugio*, were purchased from Gulf Specimen Marine Laboratories, Inc. (Panacea, FL, USA) and maintained in aerated aquaria with artificial seawater (30-32 ppt) at room temperature with consistent light: dark cycles (20-25˚C; 12L:12D). Embryos from ovigerous, adult female shrimp were harvested and transferred to small nursery chambers (100 mL glass containers). Each chamber was supplied with oxygen and maintained in an incubator under control temperature and photoperiod (20.0˚ C; 12L: 12D) in artificial seawater (30-32 ppt).

Egg Morphology and Mass

Eggs of the grass shrimp were measured along the long and short axes of the ellipsoidal egg chronologically through previously described embryonic stages (Romney and Reiber submitted to *Crustaceana*, 2011). Linear measurements were made using an ocular micrometer on a Zeiss light microscope at a magnification of 6X. Measurements of the ellipsoidal egg include the length from rostrum to abdominal flexure as the *long axis*, and the perpendicular width as the *short axis*. The dorso-ventral and lateral spanning short axes of the egg were not differentiated due to the occurrence of equal distance in measurements. The axes were further used to calculate surface area as modeled as an ellipsoid as follows:

$$SA = 4 \cdot \pi \left(\frac{l^P s^P + l^P s^P + s^P s^P}{3} \right)^{1/P}$$

Where the variable *l* is the long axis radius and *s* is the short axis radius of the egg. The value *P* is the least relative error accounts for by symmetrical approximation known as the Thomsen's variable (Klamkin, 1971; Klamkin, 1976). The volume of the egg was calculated for an ellipsoid as follows:

$$Vol = {}^4/_3 \cdot \pi \cdot (l \cdot 2s)$$

Where again, the variable *l* is the length of the egg as the long axis and *s* is the width of the egg as the short axis (as described previously). Eggs were also weighed on an electron microbalance (0.0001 mg accuracy) (Cahn 21 automatic electrobalance, Cahn Instruments Div., Ventron Corp., Cerritos, CA, USA) before and after complete water dissection to account for egg mass with and without the water component.

Ventricular volumes and cardiac parameters

Cardiovascular development and physiological parameters including end diastolic volume (EDV), end systolic volume (ESV), ejection fraction (EF), heart rate (f_H), stroke volume (V_s) and cardiac output (V_b) were investigated for the embryonic shrimp.

Ventricular volumes including EDV and ESV were measured throughout development using a modified form of the non-invasive videomicroscopic and dimensional analysis techniques adapted from Guadagnoli *et al*, (2007). Using a stereo-microscope (Leica MZ12.5; McBain Instruments, Chatsworth, CA USA) equipped with a high speed camera (Phantom v5.1; Vision Research, Inc., Wayne New Jersey USA), recordings of cardiac contractions were made through the dorsal aspect of the shrimp. The heart was positioned posteriorly in the cephalothoracic region and imaged. Cardiac V_s was determined by advancing the images frame-by-frame until the heart reaches its maximal dimension (EDV) and then to its minimal dimension (ESV). The images were captured using a computer based video frame grabber and digitizing program, ImageJ (National Institutes of Health). The dimensions of the dorsal heart image were then measured and used in a geometric equation to calculate cardiac volumes based on the shape of the heart, (ellipsoid). From these volumes, EF and V_s were determined for each of the stages of development that were characteristic of cardiac activity.

$$Vs = \mathrm{EDV} - \mathrm{ESV}$$

$$EF = \frac{SV}{EDV} x\ 100$$

Embryonic f_H, was determined by counting the number of contractions over a 30 second time intervals and reported as beats per minute. Video recordings were through a Zeiss light microscope with a CCD mounted video camera (World Precision Instruments). A value for mean

f_H was determined for each of the embryonic stages after the onset of cardiac activity (stages VIb, VIIa, VIIb, and VIII).

Embryonic V_s was measured independently from f_H due to the need for higher quality imaging, faster sampling rates and greater magnification requirements. Mean V_b was calculated as the product of the mean V_s and mean rate of contraction (beats per minute) for each stage of development.

$$V_b = f_H \times V_s$$

Statistical Analysis

Means and standard deviations were calculated for each developmental stage (n=11) for data on long and short axes, wet weight, dry weight, water mass, surface area, volume, surface area-to-volume ratio, heart rate, stroke volume, ejection fraction, end diastolic volume, and end systolic volume. The overall effects of development on the various dependent variables were studied using one way analysis of variances (SAS, ver. 9.1). A Bonferroni *t*-test was used for pairwise multiple comparisons where a significance of $P \leq 0.05$ was found. Kruskal-Wallis test, a nonparametric analysis of variance method based on ranks was used for data that are not normally distributed where a significance of $P \leq 0.05$ was found.

RESULTS

Egg Morphology and Mass

Shrimp were collected at all embryonic stages (I-VIII) for linear measurements of the ellipsoidal egg ($n \geq 40$ per stage). Through development, embryos matured with a change in egg size along the long and short axis of the egg (Fig. 1). The long axis of the egg varied significantly with development ($P \leq 0.01$, $F = 538.41$). The long axis at stage I was 0.69 mm (±0.04) and then decreased significantly to stage II at 0.65 mm (± 0.02; $P \leq 0.05$). The long axis then remained relatively unchanged until 0.73 mm (±0.02) at stage VIb. There were significant increases in length thereafter until eclosion at stages VIb, VIIa, VIIb and VIII ($P \leq 0.05$ for each stage). The short axis of the embryo also varied significantly with development ($P \leq 0.01$). The short axis at stage I was 0.51 mm (±0.02) and then significantly decreased to 0.48 mm (±0.01; $P \leq 0.05$). The short axis then remained relatively unchanged until significantly increasing at stages IV with 0.53 mm (±0.01) and stage V with 0.54 mm (0.02; $P \leq 0.05$ for both stages). Prior to eclosion, stages VIIb and VIII also significantly increased to 0.60 mm (±0.01) and 0.63 mm (±0.02; $P \leq 0.05$ for both stages). Specifically at the initiation point of contraction at stage VIb, the long axis of the embryo was 0.77 mm (±0.003) and the short axis was 0.58 (±0.004).

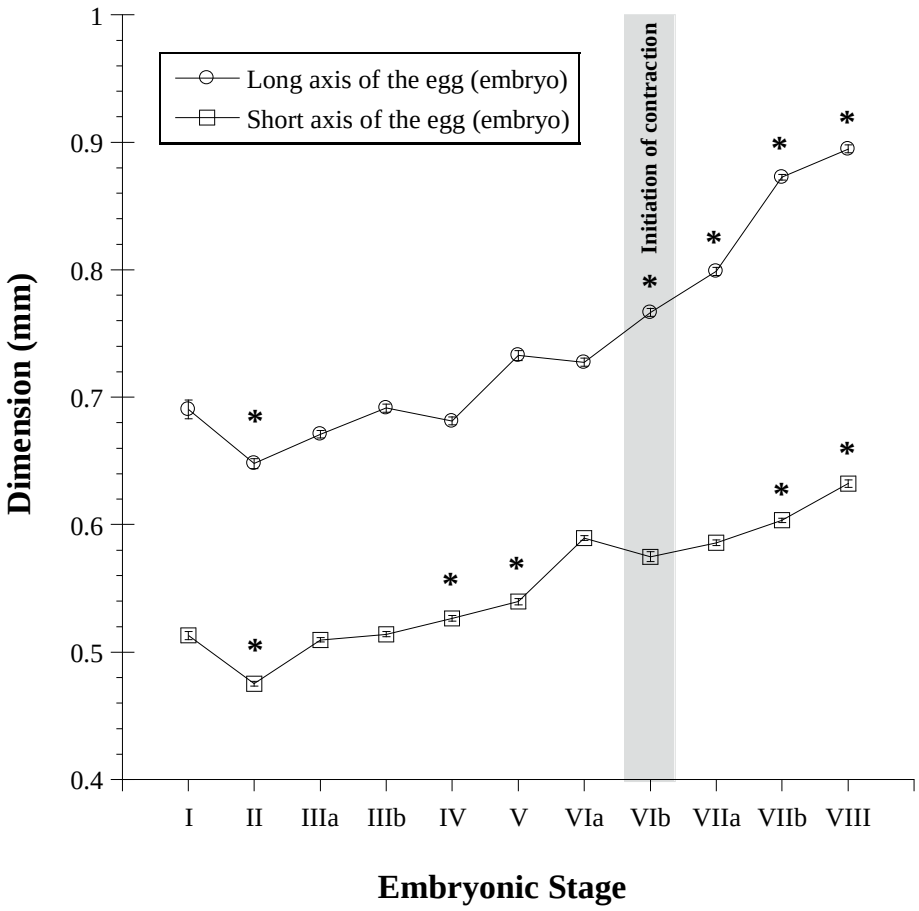

Figure 1: Linear measurements of *Palaemonetes pugio* embryos throughout development. The egg is modeled as an ellipsoid; open circles indicate the long axis of the egg and open squares indicate the short axis. * indicates significant differences from the previous level of $P \leq 0.05$. The grey bar represents the time during development when cardiac contractions are initiated. Values are shown as means ± the standard error, $n \geq 30$ at each stage.

Animals in groups of 5 were weighed and normalized for the mean mass per individual; 8 groups were analyzed for each embryonic stage ($n \geq 6$ per stage). The whole egg mass, characterized as water mass plus embryonic metabolizing tissue within the egg, is reported for each embryonic stage (Fig. 2). Dry mass was determined by subtracting the dry mass from the wet mass after complete desiccation of the embryo. This allowed one to determine embryonic water content throughout development. Whole egg mass varied significantly throughout development ($P \leq 0.01$). Mean values increased from 0.106 mg (± 0.006) at stage I to 0.241 mg

(± 0.02) at stage VIII. Interestingly, the mass of the embryo went down from 0.186 mg (± 0.03) at stage VIa, when no cardiac activity is observed, to 0.168 mg (±0.008) at VIb when cardiac contractions begin. At stage VIIa after the heart began to contract, total egg mass increased again to 0.185 mg (±0.006). Embryonic water content with development is shown in figure 2 and also varied significantly with development ($P \leq 0.01$). Between stages I to VIII, the embryonic water content increased from 0.041 mg (±0.006) composing 39% of the eggs mass at stage I to 0.18 mg (±0.018) composing 75% of the eggs mass at stage VIII. The remaining tissue after complete desiccation of the egg is also plotted against embryonic stages (Fig. 2) and varied significantly with development ($P \leq 0.01$). This mass can be referred to as the metabolizing tissue of the embryo as it is compiled from the remainder of yolk and metabolically active tissue after removal of water. Throughout development, the metabolizing mass of the whole egg ranged from 0.057 mg to 0.087 mg. Normalizing this relative to the whole egg mass, the metabolizing tissue accounted for 61% of the embryo at stage I and decreased to 24% at stage VIII.

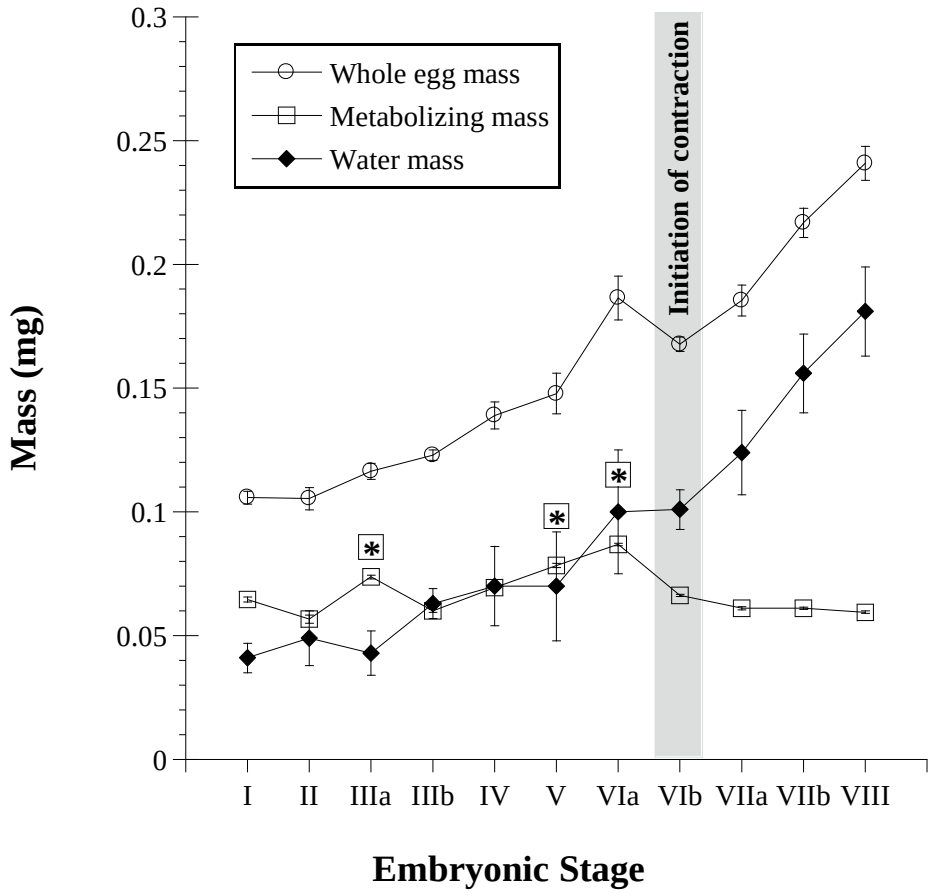

Figure 2: Whole egg mass, water composition and metabolizing mass within the chorion (mg) of *Palaemonetes pugio*. * indicates significant differences from the previous level of $P \leq 0.05$ of implied data point of respective symbol (see legend). The grey bar represents the time during development when cardiac contractions are initiated. Values are shown as means ± the standard error, $n \geq 6$ at each stage.

Linear measurements of the long and short axes of the egg allowed the calculation of surface area and volume for each individual animal ($n \leq 30$). Throughout development, surface area and volume significantly increased (Figs. 3a and b; $P \leq 0.01$). The mean surface area for embryonic shrimp at stage I was 1.023 mm^2 (±0.045) and increased to 1.615 mm^2 (±0.057) at stage VIII. The egg volume at stage I was 0.095 mm^3 (±0.006) and 0.187 mm^3 (±0.011) at stage VIII. The rate of increase for the egg surface area is much larger than the rate of increase for the volume of the egg: the mean slopes of the lines are 0.065 mm^2 and 0.01 mm^3 per developmental

stage, respectively. The surface area-to-volume ratio significantly decreased overall with development (Fig. 3c; $P \leq 0.01$). The surface area-to-volume ratio was 10.8 at stage I, and was followed by a significant increase to 11.6 at stage II ($P \leq 0.05$). After stage VIb, the ratio significantly decreased at stages VIIa, VIIb, and further to 8.6 at stage VIII ($P \leq 0.05$). The slope of the line for the surface area-to-volume ratio over developmental time is -0.26.

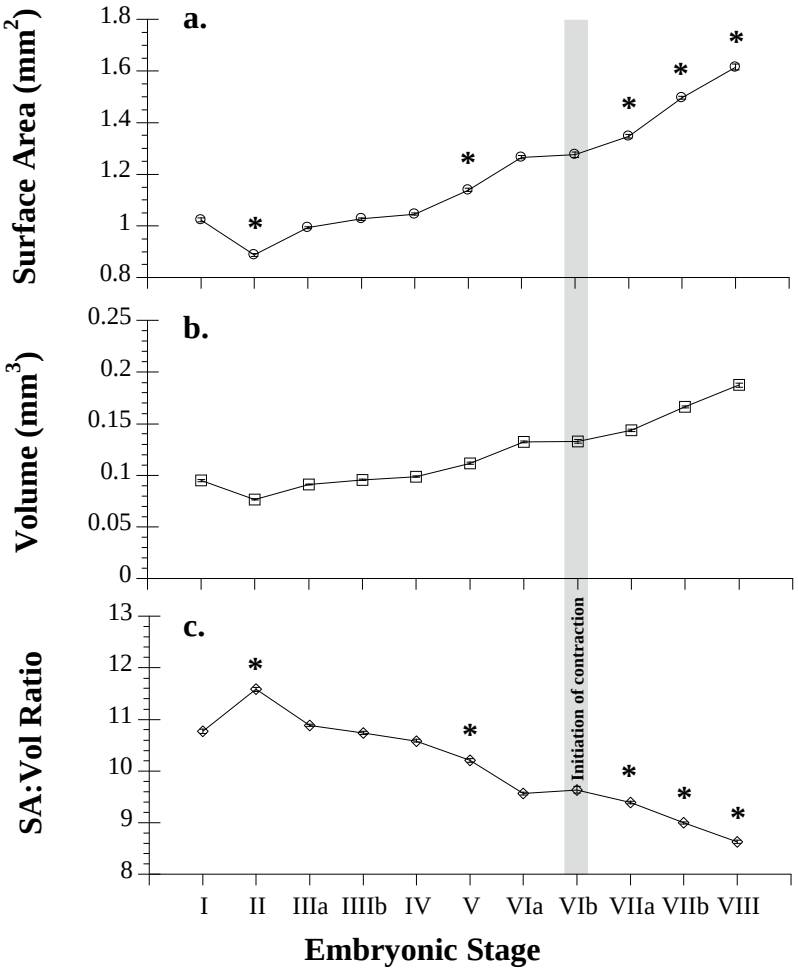

Figure 3: Surface area, volume, and the surface area-to-volume ratio calculated from linear measurements of the embryonic egg (*Palaemonetes pugio)*. * indicates significant differences from the previous level of $P \leq 0.05$. The grey bar represents the time during development when cardiac contractions are initiated. Values are shown as means ± the standard error, $n \geq 30$ at each stage.

Ventricular volumes and cardiac parameters

Cardiac contractions consistently began in the embryonic grass shrimp by stage VIb of development which was consistent with previous work (Romney and Reiber submitted to *Crustaceana*, 2011) and are reported as volumes (nL/beat) and beats per minute (bpm) for each stage of embryonic development (Figs. 4 and 5).

Ventricular volumes, EDV and ESV, of the embryonic heart increased significantly with development (Fig. 4a; $P \leq 0.01$, $F = 81.08$ and 71.03, respectively). Upon the initiation of cardiac contractions at stage VIb, ventricular EDV was 0.100 nL/beat (± 0.01). At stage VIIa, EDV significantly increased to 0.282 nL/beat (± 0.03; $P \leq 0.05$). Ventricular EDV remained at a constant level until stage VIII when it significantly increased to 0.333 nL/beat (0.03; $P \leq 0.05$). ESV followed a similar pattern and significantly increased from stage VIb at 0.055 nL/beat (± 0.01) to 0.164 nL/beat (± 0.02; $P \leq 0.05$) at stage VIIa. In addition, ESVs stayed relatively constant until stage VIII when there was a significant increase to 0.199 nL/beat (± 0.02; $P \leq 0.05$).

Embryonic ventricular EFs were calculated for each stage of cardiac activity (Fig. 4b) and showed no significant change throughout development. Mean values remained between 45 to 40% of total volume from the initiation of cardiac contraction until eclosion.

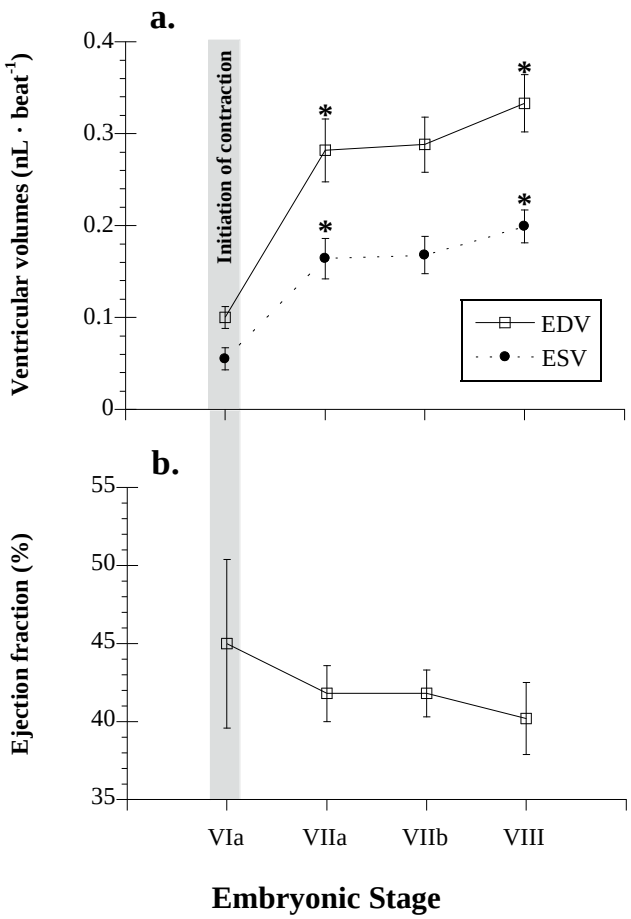

Figure 4: Ventricular volumes during the cardiac cycle of *Palaemonetes pugio*, during embryonic development. Error bars indicate the standard errors of the mean. * indicates significant differences from the previous level of $P \leq 0.05$. The grey bar represents the time during development when cardiac contractions are initiated. Values are shown as means ± the standard error, n = 6 at each stage.

Embryonic f_H and V_s increased significantly with development (Figs. 5a and b; $P \leq 0.01$). At the onset of cardiac contractions, f_H was irregular with short bursts of activity interspersed with periods of cardiac arrest. Therefore, f_H was determined by averaging over 30 second time periods. Initially at stage VIb, mean f_H was 45 bpm (±7.5) and stayed relatively constant to through to stage VIIa with 69 bpm (±12.0). At stage VIIb, f_H significantly increased to 177 bpm (±22.5; $P \leq 0.05$) and continued to onto stage VIII at 202 bpm (±33.9).

Embryonic mean V_s determined by the difference between EDV and ESV was reported for each embryonic stage (Fig. 4b). At stage VIb, the mean V_s per contraction was 0.045 nL/beat (±0.003) and was followed by a significant increase to 0.118 nL/beat (± 0.01; $P \leq 0.05$) at stage VIIa. At stage VIIb, V_s remained at constant levels with 0.120 nL/beat (± 0.01) and 0.135 nL/beat (± 0.02) at stage VIII.

Mean V_b was calculated by multiplying f_H (bpm) by V_s (nL/beat) for each of the four stages where cardiac activity was observed (Fig. 5c). Cardiac output increased from 2.0 at stage VIb to increasing to 27.2 at stage VIII.

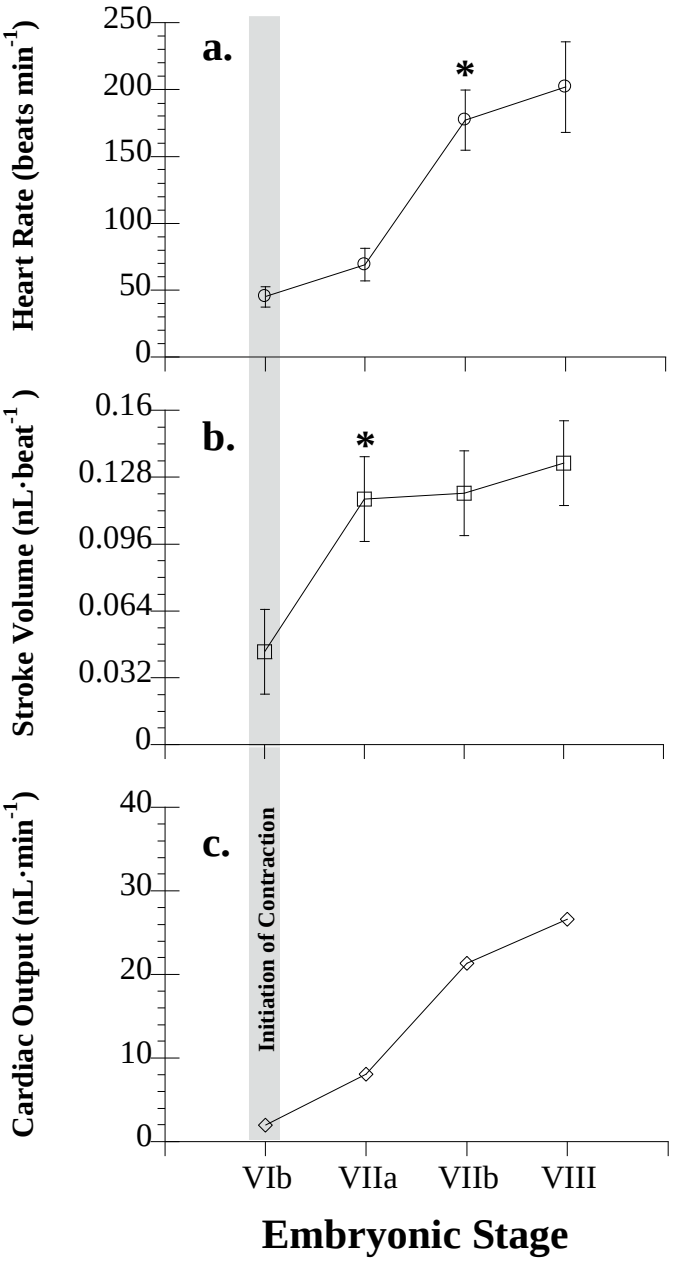

Figure 5: Ontogeny of heart rate, stroke volume, and cardiac output of embryonic *Palaemonetes pugio*. Error bars indicate the standard errors of the mean. * indicates significant differences from the previous level of $P \leq 0.05$. The grey bar represents the time during development when cardiac contractions are initiated. Values are shown as means ± the standard error, n = 6 at each stage.

DISCUSSION

Egg Morphology and Mass

The array of developmental patterns exhibited by decapod crustaceans and their ability to live and reproduce in diverse ecosystems makes this taxon useful in exploring ontogenetic processes, physiological compensatory mechanisms as well as long-term evolutionary patterns (Reiber and Harper, 2001). Decapod embryos develop externally on the maternal pleopods until eclosion and are thus directly exposed to any changes in environmental conditions (Anderson, 1973; Glas *et al*., 1997; Rabalais and Gore, 1985). This fact, results in selective pressures acting on these early life stages requiring active compensation but also potentially influencing the physiological phenotype of later developmental stages (Harper and Reiber, 2006).

We find that there is a correlation between observed plateaus (or declines) in embryonic growth parameters (including, metabolizing mass, whole egg mass, water content and dimensional growth) just prior to the onset of cardiac activity (embryonic state VIb; in all figures). At stage VIb, embryonic cardiac contractions are initiated and facilitate the movement of hemolymph within the embryo. The initiation of internal convective processes in the embryonic grass shrimp may play a role in overcoming diffusive gas exchange limitations across the chorion and allow the embryo to continue growth and maturation (Burggren and Pinder, 1991; Harper and Reiber, 2006; Reiber, 1997). The surface area (mm^2) and volume (mm^3) of the egg increase from stage I through stage VIa and then plateau until stage VIIa after which both increase (Figs. 3a and b). The surface area-to-volume ratio, calculated from egg surface area and volume, decreases steadily until stage VIa (Fig. 3c). A similar plateau to that seen in surface area and volume measurements is seen between stages VIa and VIb of mean surface area-to-volume ratios, after which shows a significant decline. The pattern discussed above is mirrored in both whole egg mass and egg water content between stages I through VIa, after which a plateau or

decline is observed (Fig. 2). Additionally, growth in both the long- and short-axis' of the egg follow a similar pattern with growth plateauing just before the heart starts to beat (Figs. 1). The fact that all of the measured growth parameters slow or decline at the same developmental stage suggests that there must be a limiting component to the system that must be overcome before growth can resume. Diffusive gas exchange limitations could result from disproportional increases in internal egg volume as compared to egg surface area. The surface area of the chorion may not be great enough to meet the increasing gas exchange demands of the metabolizing embryo, resulting in a diminution in growth. Once organized cardiac contractions begins, internal hemolymph circulation can overcome boundary layers resulting in better internal mixing, bind oxygen at the chorion for transport to deeper tissues and circulate nutrients and waste products. The result of which is to facilitate continued embryonic growth and maturation.

A more detailed examination of the surface area-to-volume ratio of grass shrimp embryos shows that there are three distinct phases of the growth curve as the embryo moves from state I through stage VIII. Regression analysis across all developmental stages shows that surface area-to-volume ratio decreases (slope of -0.26). The first phase includes a rapid increase in the surface area-to-volume ratio just post fertilization (stage 1 to stage 2) which is then followed by a steady decline through stage VIa with a slope of -0.24 (Fig. 6a). The second phase is characterized by a distinct plateau that spans stage VIa, VIb and VIIa with a slope of -0.09 (Fig. 6b). The third and last phase shows a more rapid and continued decline in the surface-to-volume ratio from stage VIIa through eclosion (stage VIII) with a slope of -0.38 (Fig. 6c). There is a positive allometric relationship between embryonic mass and metabolic activity (Burggren and Pinder, 1991). In general, body surface area-to-volume ratio and the diffusion capabilities for cutaneous gas exchange decreases with increasing mass (Burggren and Pinder, 1991). The three distinct phases of surface area-to-volume in egg growth throughout development may imply that as the embryo grows and surface area-to-volume decreases, the animal reaches a limit where further growth is

not possible through diffusive gas exchange across the chorion. At stages VIa through VIIa, the growth of the embryo decreases due to this limitation and until internal convective transport of hemolymph is initiated, growth slows. Once rhythmic and functional cardiac contractions have been established, the animal then continues to grow as by all measured parameters (Fig. 5). By introducing internal fluid convection within the egg membrane, the embryo is able to increase oxygen uptake from the environment by eliminating external boundary layers and maintaining a diffusion gradient (Harper and Reiber, 2006).

The significance of these correlations is important not only to those interested in embryonic growth and physiological ontogeny but also indicates the existence of a critical window during development that maybe highly sensitive to environmental disturbance. As the embryo makes the transition from relying solely on diffusion to meet metabolic demands to a more complex diffusion-convective system, sensory, physiological and regulatory systems must come on-line to meet the demands of the growing embryo. If the internal or external environment changes during this critical window a stress will be imposed on the embryo and a compensatory response must be elicited or the embryo maybe compromised. If the stress is severe enough in magnitude or duration the embryo may modify its systems to an extent that adult physiological or even anatomical phenotype may become modified (aka developmental plasticity).

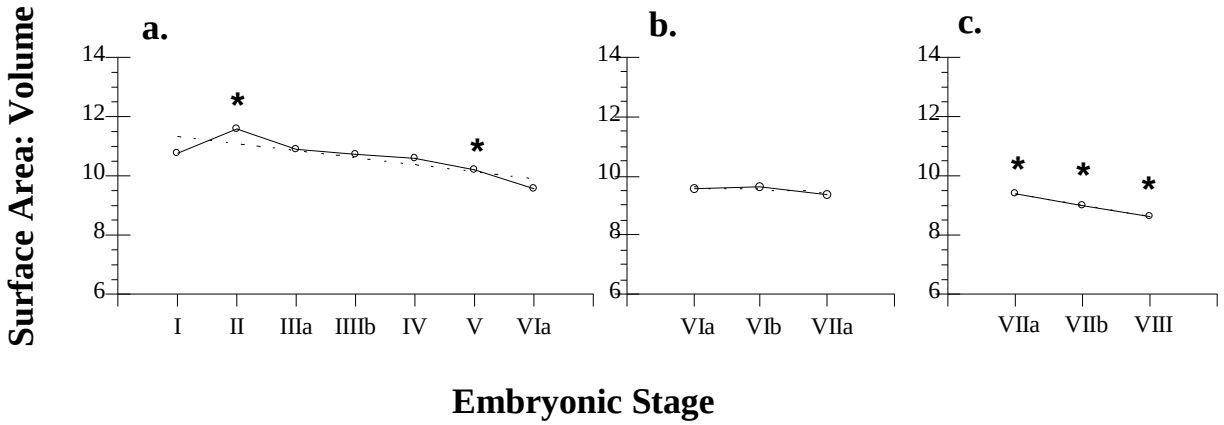

Figure 6: Three distinct phases of embryonic development, where surface area-to-volume ratio of *Palaemonetes pugio*, decreases at varying rates. (a.) Embryonic stages prior to cardiac activity with a slope of -0.24. (b.) Embryonic stages during the initiation of cardiac contractions with a slope of -0.09 (plateau phase), (c.) Embryonic stages after the heart is functional with a slope of -0.38. * indicates significant differences from the previous level of $P \leq 0.05$.

Ventricular volumes and cardiac parameters

Cardiovascular function has been studied in the adult shrimp, *Palaemonetes pugio* to further our understanding of cardiac regulatory mechanisms, energetics and environmental stress responses (Guadagnoli *et al*., 2007; Harper and Reiber, 2004). The grass shrimp is an important organism for the study of animal-environment interactions in a particularly stressful and variable estuarine habitat. The grass shrimp is a key component to estuarine ecosystems, and have been found to tolerate large ranges in temperature, salinity and dissolved oxygen (Welsh, 1975). The ecological significance of this key indicator species has also been extensively examined through environmental toxicology studies with a focus on environmental contaminants (Harper-Arabie *et al*., 2004). The cardiac responses of embryos in stages that may be sensitive to environmental stress have not been examined.

The heart of the embryonic grass shrimp, *P. pugio*, is a single muscular ventricle located in posterior end of the thorax. The heart begins to beat at stage VIb of embryonic development. Contractions are arrhythmic and lack coordination between the lateral walls of the ventricle. Vasculature supplying the anterior of the embryo beyond the aorta may exist but the primary pathway utilized is the single anteriorly extending aorta. The posterior aorta runs superficially on the abdominal segment and, like the anterior aorta, can be seen to lack valvular tight control as re-entry of suspended masses within the hemolymph occur often upon relaxation of the heart. Hemolymph appears to be circulating as indicated by the movement of these suspended masses, which is an indication of a functional cardiovascular system at this stage. In stage VIIa, contraction has become more rhythmic and regular. Cardiac development through stages VIIb and VIII continues to strengthen and it is clear the system is functional.

The basic pattern of cardiac development of *P. pugio* is similar to that seen in embryos of other decapods although comparable, most exhibit different general patterns of development (i.e. direct vs. regular development) (Harper and Reiber, 2004). At stage VIb, the heart begins to beat, after which there is a significant increase in the f_H and V_s until eclosion at stage VIII. It is common to observe an increase with f_H throughout development as it has been shown that f_H scales with body mass and both increase as the animal grows (Burggren, 1988; Burggren and Pinder, 1991; Harper and Reiber, 2006). Embryonic V_s, unlike f_H, show significant change at only once throughout stages of development with cardiac activity (VIa). This has been observed in other decapods and could likely be due to decreases in contractility over time that is offset by an increase in the dimensional growth of the cardiac chamber. From this data it is clear that V_b is dependent on V_s during stages VIb and VIIa. A transition occurs at stage VIIa where f_H plays a greater role in determining V_b. This may indicate a regulatory transition as the heart becomes more organized anatomically and with increasing metabolic demands of the growing embryo.

The timing of physiological events during embryonic development represents a relationship between the animal and its environment and the data shown here should be used in conjunction with decapods characteristic of alternative developmental trajectories. The initiation of cardiac contractions and internal convection at stage VIa will result in an increase in oxygen transport availability to tissues deeper in the developing embryo. The correlation between the observed plateau in all measured growth parameters and the on-set of cardiac activity points toward an oxygen limited embryo. Once internal convective processes are initiated growth resumes presumably because the developing embryo can now meet its metabolic demands, indicating the importance in the timing of these key physiological events.

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CHAPTER 4

CARDIAC RESPONSES TO CHANGES IN ENVIRONMENTAL SALINITY IN EMBRYOS OF *Palaemonetes Pugio*

ABSTRACT

In estuaries along the Atlantic coast of North America, daily and seasonal fluctuations in salinity occur frequently. The adult grass shrimp, *Palaemonetes pugio*, are abundant in these brackish waters and are well adapted as strict osmoregulators. As embryos, the grass shrimp lack osmoregulatory capabilities and yet are exposed to the same fluctuating salinities as adults. The effects of osmotically driven water movements are known to influence cardiovascular function in many species. However, it has not been previously investigated as to whether grass shrimp embryos exhibit cardiac compensatory responses to fluctuating environmental salinity. Such responses may serve a role in facilitating appropriate cardiac function and thus deserves examination to further our understanding of embryonic cardiac regulatory capabilities. Here we examine the ability of grass shrimp embryos to adjust cardiac parameters in response to embryonic water volume changes induced by hypersaline exposure. Embryos were transferred from control conditions (30 ppt) at defined stages of development (VIb, VIIa, VIIb, and VIII) to hypersaline water (45 ppt) and to evaluate cardiac compensatory responses. There was a significant decrease in embryonic water content, egg volume, and surface area in embryos after transfer to 45 ppt as compared to controls (30 ppt). Ventricular volumes (end diastolic and end systolic volumes) also decreased significantly with exposure and throughout development. Furthermore, heart rate and stroke volume showed a significant increase at 45 ppt during the embryonic development that may be necessary for maintenance of cardiac output in response to water volume loss. By understanding the homeostatic role of such cardiac responses to

environmental stress during embryonic development, we can infer possible mechanisms regulatory capabilities.

INTRODUCTION

The dynamic effects of salinity on circulatory function have provided the foundation for previous reviews on cardiac physiology in decapod crustaceans (Mantel and Farmer, 1983; McGaw and McMahon, 1996; Spaargaren, 1973; Spaargaren, 1974; Wheatly, 1988). Intertidal zones and estuaries are characterized by rapid fluctuations in salinity, and therefore the inhabitants of these regions are subject to a variety of osmotic stresses. When decapods are exposed to hyposmotic conditions relative to their internal hemolymph, osmotic pressures favor the movement of water into the animal and result in internal volume loading. Alternatively, when exposed to hyperosmotic conditions, animals lose water to their environment (Mantel and Farmer, 1983; Withers, 1992). In order to tolerate a broad range of salinities, many decapod crustaceans regulate their extracellular environment homeostatically to buffer against significant salinity fluctuations (Mantel and Farmer, 1983; Wheatly, 1988). Osmoregulators actively regulate the osmotic concentration of their body fluids to maintain internal water composition (Withers, 1992). To compensate for passive water and ion movement between their hemolymph and the environment, osmoregulating decapods regulate body fluid ion and water composition by integument and gill ion pumps, urine excretion from the antennal glands, and modifications in cardiovascular performance (Gilles and Pequeux, 1983; Withers, 1992).

Within the infraorder Caridea, the adult grass shrimp, *Palaemonetes pugio*, are euryhaline osmoregulators, and maintain their hemolymph osmotic concentration between 500 to 650 mOs/kg H_2O in ranges of seawaters between 60 to 925 mOs/kg H_2O, respectively (Mantel and Farmer, 1983; Roesljadi *et al*., 1976). In addition to their osmoregulatory abilities,

environmental salinity has an indirect effect on their cardiac function mediated by changes in hemolymph volume (McMahon, 1999; Wheatly, 1988). Hemolymph volume changes resulting from osmotically induced water movements have been shown to occur in adult decapod crustaceans, but little is known about these effects during embryonic and larval periods. Modifications in hemolymph volume might reflexively stimulate changes in heart rate and stroke volume that stabilize cardiac function (Burggren *et al*., 1990; McGaw and McMahon, 1996; McGaw and Reiber, 1998; Sabourin, 1984; Spaargaren, 1973; Ungherese *et al*., 2008).

Cardiac compensatory responses in crustaceans may result from environmental stresses to facilitate adequate oxygen delivery to metabolizing tissues (Bakhmet *et al*., 2005; DeFur and Mangum, 1979). Specifically, cardiac parameters (e.g. heart rate and stroke volume) can be adjusted independently in crustaceans when exposed to stresses such as temperature, salinity and hypoxia (McMahon and Wilkens, 1983; Reiber and Harper, 2001; Wilkens, 1999). The globular heart of decapod crustaceans is a single chambered ventricle suspended within a pericardial sinus by suspensory ligaments. Upon systolic contraction of the myocardium, oxygenated hemolymph is forced out of the heart through the coordinated opening of ostia into peripheral arterial outlets. The hemolymph supplies metabolically active tissues then collects in paired infrabranchial sinuses that lead to the gills. From the gills, re-oxygenated hemolymph moves through the branchio-cardiac veins to the pericardial sinus and back into the heart. The heart then returns to a presystolic volume which draws the hemolymph back through multiple pairs of ostia leading into the ventricle of the heart aided by both the hemolymph pressure gradient and the rebound of the suspensory ligaments (Guadagnoli *et al*., 2007; Reiber and McGaw, 2009).

The initiation of cardiac contractions (or cardiac activity) occurs during stage VIb of the embryonic developmental period in grass shrimp (Romney and Reiber submitted to *Crustaceana* 2011). At this time, embryos are encased within a chorion and attached to the abdominal pleopods of female shrimp. Embryos are carried externally, and as such they are exposed to

temporal and spatial changes in salinity associated with the intertidal and estuarine habitats yet, unlike the adults, palaemonid embryos lack the branchial and excretory organs responsible for osmoregulation (Anderson, 1982; Anderson, 1985; Ituarte *et al*., 2005; Taylor and Seneviratna, 2005). We hypothesize that *P. pugio* embryos exposed to hypersaline water (45 ppt), will experience an osmotic water loss. This will further result in a decrease in venous return to the heart and a decrease in ventricular stretch thus impacting stroke volume and ultimately decreasing cardiac output and oxygen delivery to the tissues. To compensate for the reduction in hemolymph return to the ventricle, heart rate and stroke volume will increase to maintain the cardiac output necessary to sustain aerobic metabolism and growth. Based on this data we can infer embryonic cardiac regulatory capabilities that allow these early stage embryos to successfully develop in the face of such fluctuating salinities.

MATERIALS AND METHODS

Animal culture

Adult grass shrimp, *P. pugio*, were purchased from Gulf Specimen Marine Laboratories, Inc. (Panacea, FL, USA) and maintained in 40 L aerated aquaria with artificial seawater at room temperature with consistent light and dark cycles as control conditions (30-32 ppt; 20-25˚C; 12L:12D). Embryos from ovigerous, adult female shrimp were harvested and transferred to small nursery chambers (approximately 100 mL glass jars). Each chamber was supplied with room air and maintained in an incubator under the same control conditions (30-32 ppt; 20-25˚C; 12L: 12D).

Experimental Design

Grass shrimp eggs were transferred from control conditions (30 ppt) to hypersaline (45 ppt) treatment chambers (also in 100 mL glass jars) to evaluate changes in egg morphometrics and cardiac physiology during late embryonic stages of development. Stages of development were previously described by Romney and Reiber (submitted to *Crustaceana*, 2011). Stage VIb was defined as the point of development where cardiac contractions begin, and stages VIIa, VIIb, and VIII were defined by morphological development of the eye: linear pigmentation at VIIa, oval pigmentation at VIIb, and the circular eye at VIII. Egg morphometrics and cardiac parameters were measured after a two hour acclimation period (post-transfer time) to hypersaline water (45 ppt). As preliminary data to determine when cardiac parameters were stable, intermediate time periods of 10, 30, 60, and 120 minutes were chosen to collect heart rates from embryos at each development stage VIb, VIIa, VIIb, and VIII after being transferred from 30 ppt to 45 ppt (Fig. 1). A one way analysis of variance showed no statistical difference in heart rates ($P \leq 0.05$) at any time period between 10 and 120 minutes (2 hours) for stages VIb, and VIIa, and VIIb. For consistency, all stages of development were determined to have stable cardiac parameters between initial transfer time of 10 minutes until 2 hours post-transfer. To control for stress induced by the physical transfer, embryos that were used for analysis under control conditions were transferred from 30 ppt to nursery chambers also maintained at 30 ppt and allowed to acclimate for 30 minutes prior to data collection.

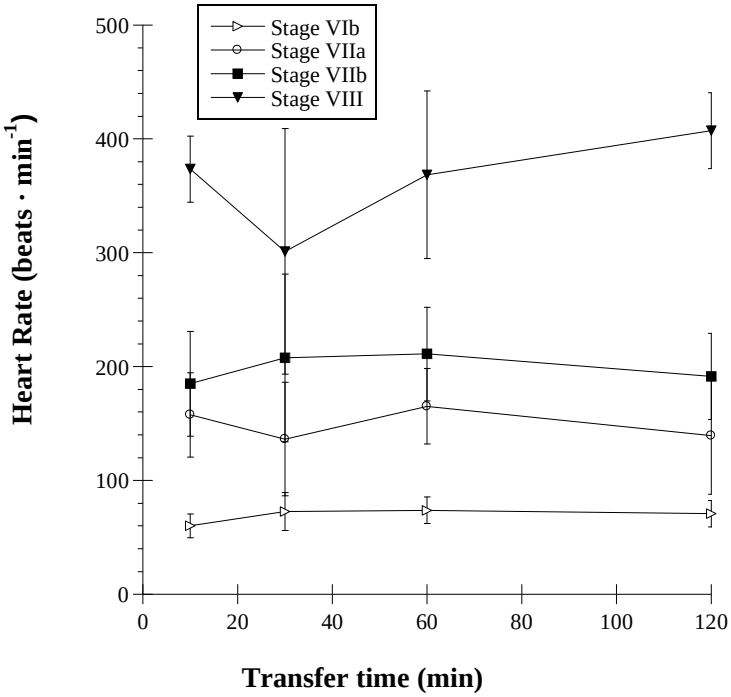

Figure 1: Embryonic heart rates of *Palaemonetes pugio*, Holthius, 1949, after being transferred from 30 ppt salinity to 45 ppt salinity at time periods 10, 30, 60, and 120 minutes to determine when cardiac parameters were stable. Stages VIb, VIIa, and VIIb showed no significant difference in heart rate over the intermediate time periods.

Egg morphometrics

Eggs of the grass shrimp were measured along the short and long axes of the ellipsoidal egg chronologically through previously described embryonic stages based on whole egg morphology as previously mentioned (Romney and Reiber submitted to *Crustaceana*, 2011). Linear measurements were made using an ocular micrometer on a Zeiss light microscope at a magnification of 6X. Measurements of the ellipsoidal egg included the length from rostrum to abdominal flexure as the long axis, and the perpendicular width as the short axis. Either the dorso-ventral or the lateral spanning axes of the egg were used as the short axes due to the

occurrence of their equal distances in measurements. The long and short axes were used to calculate surface area modeled as an ellipsoid as follows:

$$SA = 4 \cdot \pi \left(\frac{l^P s^P + l^P s^P + s^P s^P}{3} \right)^{1/P}$$

where *l* is the long axis radius and *s* is the short axis radius of the egg. The value *P* is the least relative error accounted for by symmetrical approximation known as the Thomsen's formula (Klamkin, 1971; Klamkin, 1976). The linear axes were also used to calculate the volume of the egg modeled as an ellipsoid as follows:

$$Vol = {}^4/_3 \cdot \pi \cdot (l \cdot 2s)$$

where *l* is the length of the egg as the long axis and *s* is the width of the egg as the short axis. Values of surface area and volume were then further used to determine the surface area-to-volume ratio.

Embryonic water content

Grass shrimp eggs that were treated under control (30 ppt, 30 minutes) and experimental (45 ppt, two hours) conditions were removed from their chambers and weighed on an electron microbalance (0.0001 mg accuracy; Cahn 21 automatic electrobalance, Cahn Instruments Div., Ventron Corp., Cerritos, CA, USA) in groups of 5. Groups of five eggs were measured together and averaged for the wet mass of an individual egg. Following wet mass measurements, eggs were immediately transferred to a desiccation oven to evaporate embryonic water (approximately 24 hours). After complete desiccation, (as established by sequential measurements of a constant mass) each group of five eggs was again weighed in order to calculate the average dry mass per individual that corresponds to the metabolizing tissue of the embryo.

Ontogeny of Heart Rate, Stroke Volume, and Cardiac Output

Embryonic cardiac parameters were measured under both control and experimental conditions after a two hour post-transfer acclimation period (20-25˚C; 30 and 45 ppt, respectively) at development stages VIa, VIIa, VIIb, and VIII. Embryos were imaged microscopically using a stereo-microscope (Leica MZ12.5; McBain Instruments, Chatsworth, CA USA) equipped with a high speed camera (Phantom v5.1; Vision Research, Inc., Wayne New Jersey USA). Recordings of cardiac contractions from the dorsal and lateral aspects of the heart through the thoracic region of the animal were captured and analyzed with an image processing program, ImageJ (National Institutes of Health). Ventricular volumes including end diastolic (EDV) and end systolic volumes (ESV) were marked on still frames when the ventricle was at its most maximal and minimal dimensions, respectively as a modified form of the non-invasive video-microscopic and dimensional analysis techniques adapted from Guadagnoli *et al*, (2007). The dimensions of the heart were then used in a geometric equation to calculate cardiac volumes based on the shape of the heart, (ellipsoid; same formula as previously used for egg volume) and calculated as nanoliters per contraction ($nL \cdot beat^{-1}$). The maximal dimension or EDV corresponds to the volume of hemolymph that enters the ventricle prior to contraction and is referred to as preload. The minimal dimension or ESV is the volume of hemolymph that remains in the ventricle after contraction. The two values, EDV and ESV, can be further used for the calculation of cardiac stroke volume (V_s), the volume of hemolymph that is ejected from the ventricle during contraction, and the ejection fraction (%; EF), the fraction of ejected hemolymph volume:

$$V_S = \mathrm{EDV} - \mathrm{ESV}$$

$$EF = \frac{V_S}{EDV} x\ 100$$

Embryonic f_H, was determined as beats per minute by counting the number of contractions over 30 second intervals. Video recordings were made independently from the previous recordings of V_s using a Zeiss light microscope with a CCD video camera (World Precision Instruments). A value for mean f_H was determined for each of the embryonic stages (VIb, VIIa, VIIb, and VIII), at either 30 ppt and 45 ppt after a two hours post-transfer time from control conditions (30 ppt). Mean embryonic cardiac output, V_b, was calculated as the product of the mean V_s per mean f_H, (nL·min^{-1}), for each stage of cardiac activity for either salinity treatment (30 and 45 ppt).

$$V_b = f_H \times V_s$$

Statistical Analysis

The overall effects of development on the various dependent variables were studied using one way analysis of variances (SAS, ver. 9.1) for the values of wet mass, water mass, surface area, volume, surfacea area-to-volume ratio, EDV, ESV, f_H, V_s, and EF to detect differences among development stage VIb, VIIa, VIIb, and VIII. At each developmental stage, a Student's *t*-test was used to further compare various measurements between salinity level of 30 and 45 ppt. A Bonferroni *t*-test for pairwise multiple comparisons was used in order to correct for the effect of multiple corrections with a significance level of $P \leq 0.05$. Also, a Kruskal-Wallis test was used as a nonparametric analysis of variance method based on ranks for data that were not normally distributed (even after transformation).

RESULTS

Embryonic water composition

Whole egg mass was determined for individual eggs at each embryonic stage (VIb, VIIa, VIIb, VIII) after being transferred to control (sham transfer at 30 ppt, n = 8) and treatment salinities (45 ppt; n = 8). Mean egg mass for embryos transferred to 45 ppt weighed significantly less ($P \leq 0.05$) than embryos at 30 ppt at stages VIb, VIIa, and VIIb (Fig. 2) . Mean embryonic wet mass increased significantly throughout development at stages VIIb and VIII in 30 ppt and stages VIIa and VIII at 45 ppt ($P \leq 0.01$ for each stage with significant change).

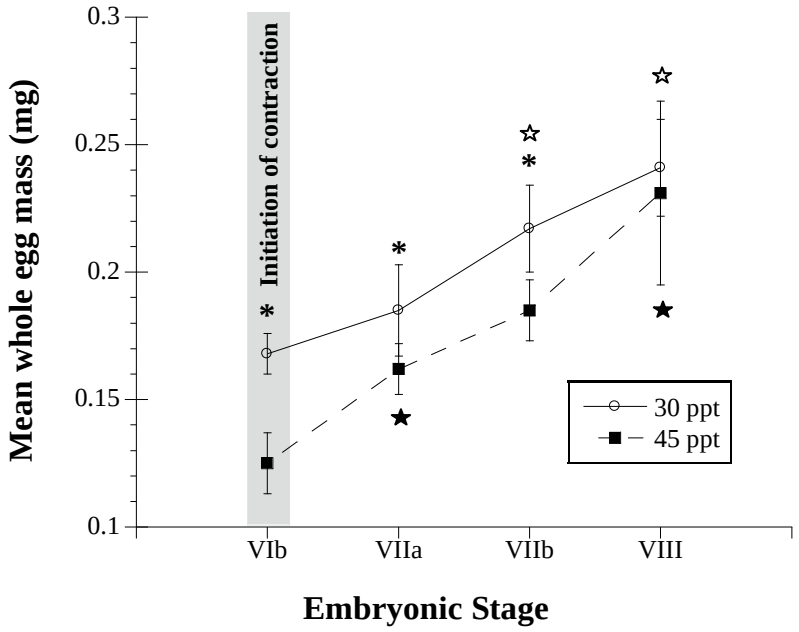

Figure 2: Individual egg mass of *Palaemonetes pugio* embryos during development at 30 ppt (circles) and 45 ppt after being transferred for two hours (squares) from 30 ppt. * indicates a significant difference between the variable at either salinity of the same stage as determined by *t*-test with $P \leq 0.01$. The open and solid stars represent stages that are significantly different from the previous stage at each salinity level (30 and 45 ppt, respectively). The grey bar represents the time during development when cardiac contractions are initiated. The values shown are the means ± the standard error, n = 8 at each stage for both salinities.

After complete desiccation, the same embryos were re-measured to determine individual embryonic water content (1 µL of H_2O has a mass of 1 mg). Mean embryonic water volume was calculated for each development stage after the heart began contracting (Fig. 3). There was a significant decrease in embryo water content in eggs exposed to 45 ppt salinity as compared to control embryos 30 ppt ($p = 0.05$) at stages VIb, VIIa, and VIIb. Initially, at stage VIb, embryonic water volume at 30 ppt was 0.101 µL (± 0.01) and 0.077 µL (± 0.01) at 45 ppt. At stage VIIa, embryos at 45 ppt had less embryonic water with 0.102 µL (± 0.02) than those at 30 ppt with 0.124 µL (± 0.02). At stage VIIb, embryos at 45 ppt had significantly less embryonic water with 0.116 µL (±0.01) than embryos at 30 ppt with 0.156 µL (±0.02). Yet at stage VIII, the mean embryonic water volume in embryos transferred to 45 ppt was not significantly different than the mean at 30 ppt. Before eclosion, embryonic water volume at stage VIII was 0.181 µL (±0.02) at 30 ppt and was 0.170 µL (±0.03) at 45 ppt. Mean embryonic water composition increased significantly at both salinities throughout stages VIIa, VIIb and VIII at 30 ppt and at stage VIII at 45 ppt ($P \leq 0.01$ for all significant changes).

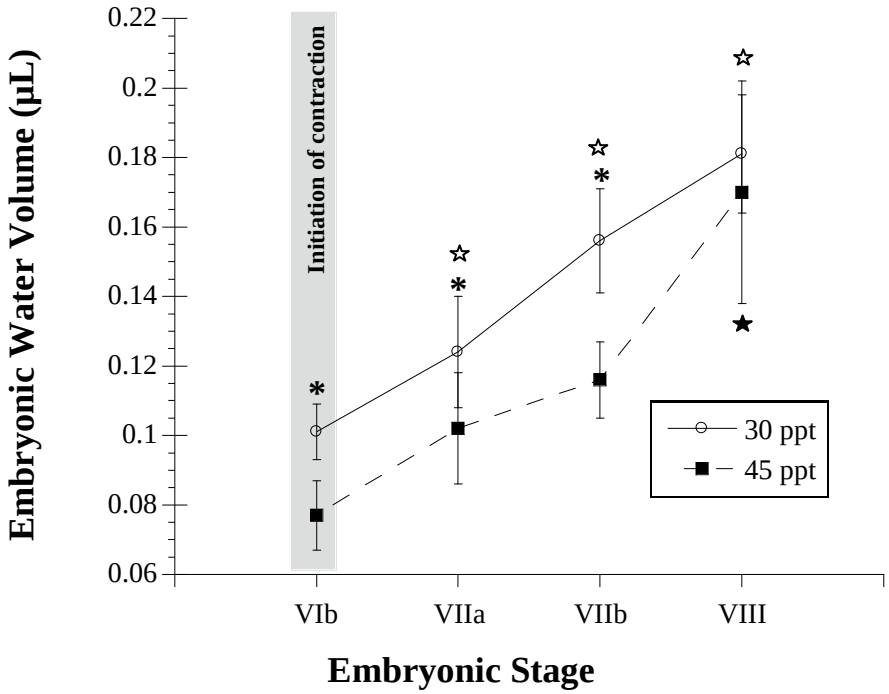

Figure 3: Embryonic water volume within the eggs of *Palaemonetes pugio*, during development at 30 ppt (circles) and then after being transferred to 45 ppt for two hours (squares). * indicates a significant difference between the variable at either salinity of the same stage as determined by *t*-test with $P \leq 0.05$. The open and solid stars represent stages that are significantly different from the previous stage at each salinity level (30 and 45 ppt, respectively). The grey bar represents the time during development when cardiac contractions are initiated. Values are shown as means ± the standard error, n = 8 at each stage for both salinities.

Egg morphometrics

Based on previous egg dimensions, surface areas (mm^2) and whole egg volumes (mm^3) were calculated for each developmental stage at both 30 and 45 ppt (n = 40; Figs. 4a and 4b). Both egg surface area and volume increased throughout stages of development at both salinity treatments ($P \leq 0.01$). The surface of the egg showed an average increase in area (mm^2) of 1.8% from stages VIb to VIII at 30 ppt and 2.7% at 45 ppt. At stage VIb the surface area was significantly less at 45 ppt with 1.28 mm^2 ± 0.07 compared to the mean value at 30 ppt with 1.08 mm^2 ± 0.03 at 45 ppt. At stage VIII, the mean surface areas of the egg at either salinity were not

significantly different (1.62 mm^2 ± 0.07 at 30 ppt and 1.60 mm^2 ± 0.05 at 45 ppt). Mean whole egg volume was significantly larger at 30 ppt for stage VIb compared to the mean egg volume at 45 ppt (1.33 mm^3 ± 0.01 and 0.103 mm^3 ± 0.01, respectively). At stage VIII mean egg volumes were not significantly different at either salinity (0.187 mm^3 ±0.01 and 0.186 mm^3 ± 0.01, respectively). Mean whole egg volumes at 30 ppt had a positive slope of 1.8 % throughout developmental stages and at 45 ppt had a slope of 2.7%. The mean surface area-to-volume ratios were calculated for the eggs at each stage and were found to decrease significantly for either salinity from VIb to VIII ($P \leq 0.01$; Fig. 4c). At 30 ppt the surface area-to-volume ratio decreased from 9.6 to 8.6 from stage VIb to VIIII. Surface area-to-volume ratios of eggs transferred to 45 ppt at each stage decreased from 10.4 at stage VIb to 8.6 at stage VIII.

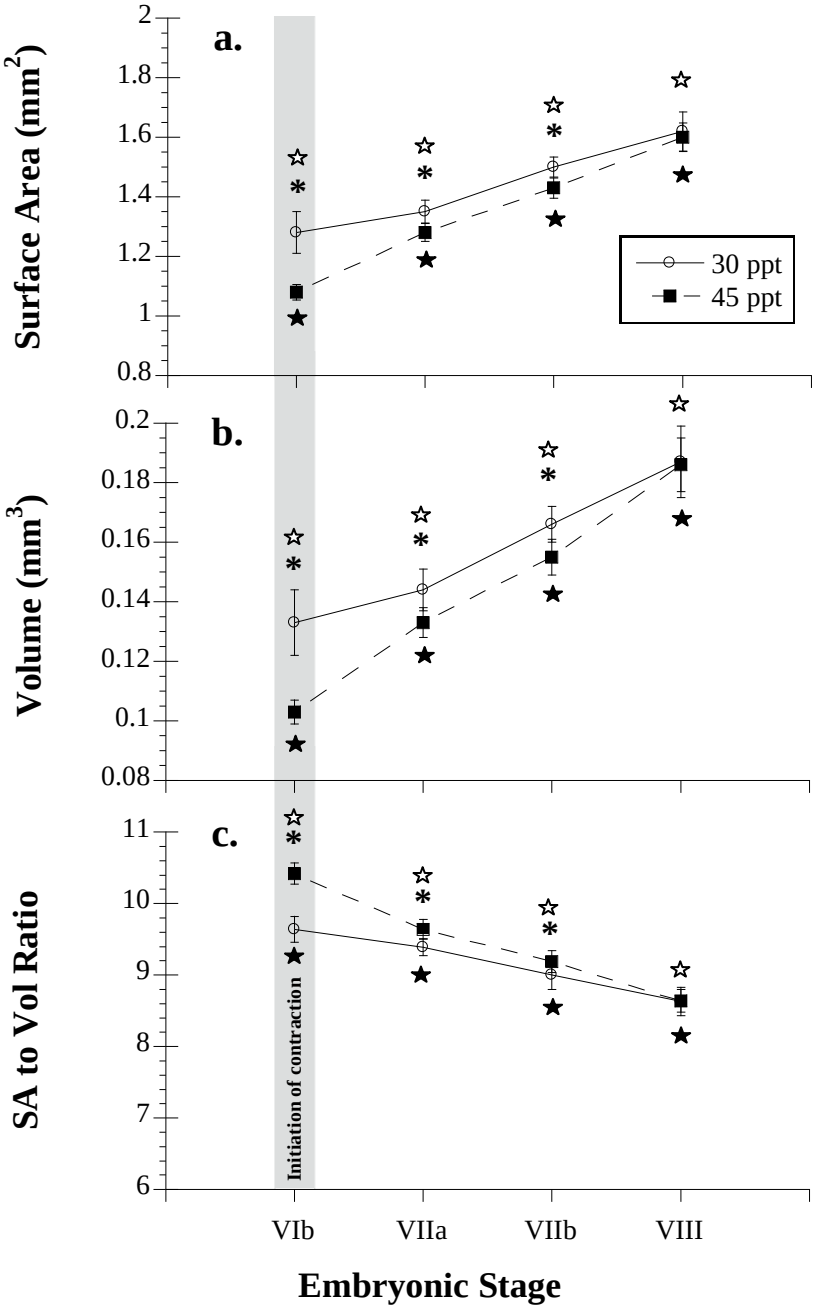

Figure 4: Embryonic surface area (a.), volume (b.) and surface area to volume ratio (c.), per embryonic stage of *Palaemonetes pugio* at 30 ppt salinity (circles/solid line) and 45 ppt (squares/dashed line). * indicates a significant difference between the variable at either salinity of the same stage as determined by *t*-test with $P \leq 0.05$. The open and solid stars represent stages that are significantly different from the previous stage at each salinity level (30 and 45 ppt, respectively). The grey bar represents the time during development when cardiac contractions are initiated. Values are shown as means ± the standard error, n = 8 at each stage for both salinities.

Ontogeny of Heart Rate, Stroke Volume, and Cardiac Output

After initiation of cardiac activity (stage VIb), the mean EDV ($nL \cdot beat^{-1}$) and ESV ($nL \cdot beat^{-1}$) were determined for each embryonic stage (VIb, VIIa, VIIb, and VIII) two hours after being transferred from 30 ppt to either 30 or 45 ppt (Figs. 5). As embryos developed, there was a significant increase in EDV at stages VIIa and VIII at both 30 and 45 ppt ($P \leq 0.01$). There was also a significant increase throughout development in ESV at stages VIIa and VIII when embryos were transferred to either salinity treatment ($P \leq 0.05$). At stage VIIa, EDV was significantly less for embryos transferred to 45 ppt compared to embryos at 30 ppt (0.240 ± 0.024 and 0.262 ± 0.015 $nL \cdot beat^{-1}$, respectively; $P \leq 0.05$). During the same stage, mean ESV was also significantly less for embryos transferred to 45 ppt with 0.155 ± 0.017 $nL \cdot beat^{-1}$ compared to embryos transferred to 30 ppt with 0.162 ± 0.008 $nL \cdot beat^{-1}$ ($P \leq 0.05$). Mean EDV for embryos at stage VIII who were transferred to 45 ppt were significantly lower with 0.347 ± 0.037 $nL \cdot beat^{-1}$ compared to embryos transferred to 30 ppt with 0.358 ± 0.020 $nL \cdot beat^{-1}$ ($P \leq 0.05$). However, mean ESV for embryos at stage VIII had an opposite effect of what was previously seen at 45 ppt with 0.222 ± 0.026 $nL \cdot beat^{-1}$ which was significantly higher compared to embryos at 30 ppt with 0.208 ± 0.019 $nL \cdot beat^{-1}$; ($P \leq 0.05$).

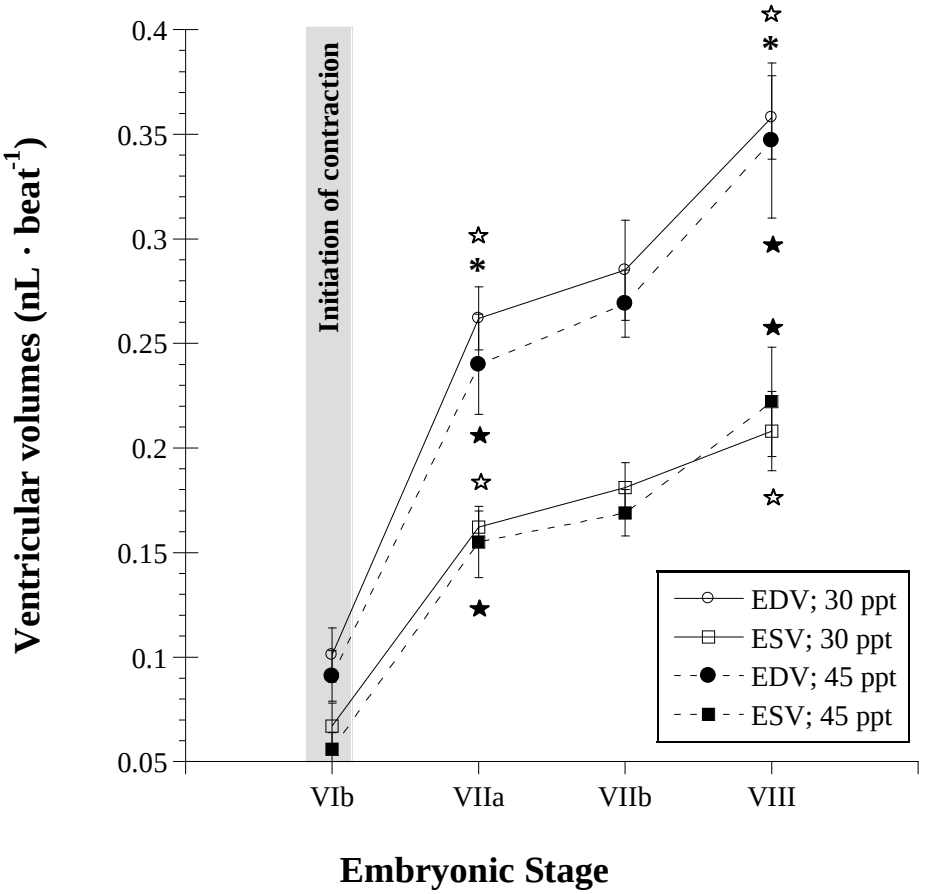

Figure 5: End diastolic (squares) and systolic (circles) volumes of *Palaemonetes pugio* embryos at 30 ppt (open) and 45 ppt (closed) salinities. * indicates a significant difference between the variable at either salinity of the same stage as determined by *t*-test with $P \leq 0.05$. The open and solid stars represent stages that are significantly different from the previous stage at each salinity level (30 and 45 ppt, respectively). The grey bar represents the time during development when cardiac contractions are initiated. Values are shown as volume means ± the standard error, n = 6 at each stage for both salinities.

The mean f_H (bpm) was determined for each of the following embryonic stages two hours after being transferred from 30 ppt to either 30 or 45 ppt (Fig. 6a). At stage VIb, the embryonic f_H was initially sporadic and became more synchronized and rhythmic by stage VIIa. Mean f_H increased significantly at 30 ppt for stages VIIb and VIII and at 45 ppt at stage VIII ($P \leq 0.01$). Throughout development, f_H was significantly higher for embryos at 45 ppt than the f_H of embryos at 30 ppt at all four stages ($P \leq 0.05$). At stage VIb, embryos at 30 ppt had a mean f_H of

53 bpm ±10, which was not significantly different from that of embryos at 45 ppt with 73 bpm ±17. At stage VIII, mean f_H of embryos at 30 ppt increased to 213 bpm ±57 which was significantly higher than the mean f_H of embryos at 45 ppt of 301 bpm ±108 ($P \leq 0.05$).

Mean cardiac V_s (nL·beat^{-1}) was calculated from individual EDV and ESV for embryos transferred to 30 and 45 ppt (Fig. 6b). Throughout development, there was a significant increase at stages VIIa and VIII at either salinity treatment ($P \leq 0.01$). At those stages, mean V_s of embryos at 45 ppt was significantly higher than for embryos at 30 ppt ($P \leq 0.05$ for both stages). At 30 ppt, mean embryonic V_s increased from 0.034 nL·beat^{-1} ± 0.002 at stage VIb to 0.15 nL·beat^{-1} ± 0.016 at stage VIII. At 45 ppt, mean embryonic V_s increased from 0.056 nL·beat^{-1} ± 0.005 to 0.222 nL·beat^{-1} ±0.015.

Embryonic V_b (nL·min^{-1}) for embryos transferred to 30 and 45 ppt was calculated from the mean f_H and V_s for each developmental stage and therefore cannot be analyzed for significant differences (Fig. 6c). At stage VIb, the mean V_b for embryos transferred to 30 ppt was 18 nL·min^{-1} and at stage VIII was 32 nL·min^{-1}. At 45 ppt, V_b was 4.1 nL·min^{-1} at stage VIb and 67 nL·min^{-1} at stage VIII.

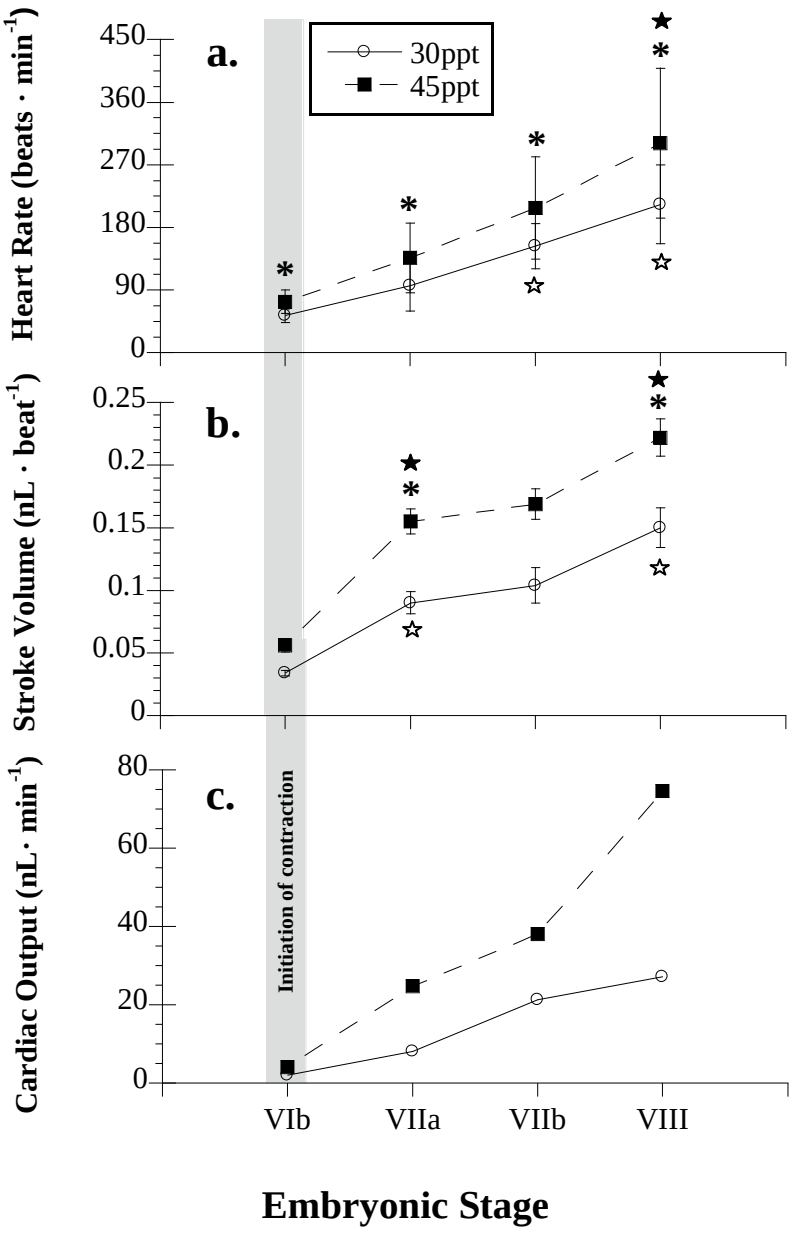

Figure 6: Embryonic heart rate (a.), stroke volume (b.) and cardiac output (c.) of *Palaemonetes pugio* at both 30 ppt (circles/solid line) and then transferred to 45 ppt (squares/dashed line) for two hours. * indicates a significant difference between the variable at either salinity of the same stage as determined by *t*-test with $P \leq 0.05$. The open and solid stars represent stages that are significantly different from the previous stage at each salinity level (30 and 45 ppt, respectively). The grey bar represents the time during development when cardiac contractions are initiated. Values are shown as the means ± the standard error, n = 6 at each stage for both salinities.

Ejection fraction (EF) is the fraction of hemolymph ejected from the ventricle during one cardiac cycle and was determined for all four stages of development from individually recorded values of mean V_s and EDV at control (30 ppt) and experimental (45 ppt) conditions (Fig. 7). Throughout development, embryos at 30 ppt showed no significant difference in mean EF from stage VIb to VIIb. However, at stage VIII, there was a significant increase in EF (41.9% ± 3.9%; $P \leq 0.05$). There was no significant increase in EF from stage VIb to VIII for embryos transferred to 45 ppt. At both stages VIIa and VIII, the mean EF at 30 ppt was significantly higher than the mean EF at 45 ppt ($P \leq 0.01$).

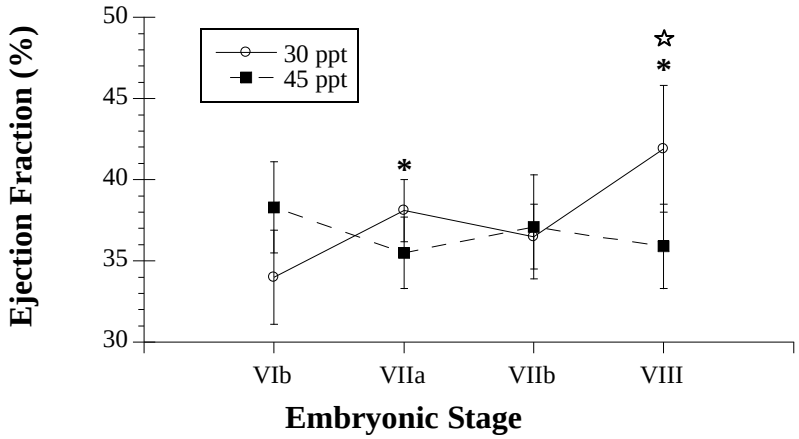

Figure 7: Ejection fraction of ventricular volume during embryonic development of *Palaemonetes pugio*, at both 30 ppt (circles/solid line) and then transferred to 45 ppt (squares/dashed line) for two hours. * indicates a significant difference between the variable at either salinity of the same stage as determined by *t*-test with $P \leq 0.05$. The open star represents the stage that was significantly different from the previous stage at 30 ppt. The grey bar represents the time during development when cardiac contractions are initiated. Values are shown as mean percents ± the standard error, n = 6 at each stage for both salinities.

DISCUSSION

In the current study, embryos of the grass shrimp, *P. pugio*, were successfully reared under control (30 ppt) and experimental (45 ppt) conditions through developmental stages with

cardiac activity (stages VIb, VIIa, VIIb, and VIII) to eclosion. Shrimp embryonic development was characteristic of what has been previously described by Romney and Reiber (submitted to *Crustaceana*, 2011). After being transferred from control conditions to 45 ppt for two hours at each developmental stage, embryos exhibited a decrease in whole egg mass and water volume that can be largely attributed to embryonic water loss to the environment (Figs. 2 and 3). In addition, there was a decrease in egg surface area and egg volume after being transferred to hypersaline water (45 ppt; Figs. 4a and b). It is evident here that when exposed to high salinity during embryonic development, there is an osmotic water loss to the environment through the permeable coat of the egg. This supports previous studies on the water and solute exchange of the chorion for the grass shrimp embryo (Glas *et al*., 1997).

Throughout development when embryos were transferred to a higher salinity, there was an observed decrease in EDV and ESV (Fig. 5). This is likely due to a decrease in hemolymph volume that occurred as a result of embryonic water loss. Thus, if hemolymph volume was reduced through water loss to the environment, it is highly probable that a concomitant decrease would occur in venous hemolymph return to the heart.

At all four stages of development, there was an increase in f_H for embryos transferred to hypersaline water (Fig. 6a). With a decrease in hemolymph volume within the circulatory system, an increased f_H may compensate for the reduced preload of the ventricle. While individual ventricular volumes, EDV and ESV were significantly lower at 45 ppt, there was an overall increase in the difference between the two volumes, cardiac V_s (Fig. 6b). This suggests that embryonic water loss caused a reduction in the volume of hemolymph that returned to the heart for each cardiac cycle and such decreases in ventricular V_s caused an elevated f_H to maintain cardiac output within the circulatory system. This is further supported by the sustained levels of EF at both 30 and 45 ppt. It is important for these embryos to sustain cardiac output to maintain the aerobic metabolism of actively growing tissues.

Maintenance in V_b in response to changes in hemolymph volume can serve as a compensatory reflex that is synonymous to the vertebrate baroreflex response and may be explained by the Frank-Starling Law of the heart (Bagshaw, 1985; Burggren *et al*., 1990). Here, the length-tension relationship of cardiac muscle tissue regulates both cardiac stroke volume and systolic pressure. An increase in hemolymph volume will increase venous return to the heart which in turn would result in an expansion of the ventricle. The increase in ventricular pre-loading volume (EDV) will shift the length tension relationship of the myofibril arrangement, where a greater tension can be generated by the myocardium. The overall effect produces an increase in hemolymph pressure through an increase in cardiac contractility. Conserved cardiac EF between salinity treatments could likely be a cardiac reflex to hemolymph volume changes and may suggest that transition of active regulation of f_H may occur during embryonic period of development in the grass shrimp.

While adult grass shrimp are euryhaline and can survive large shifts in salinities, the larvae are less tolerant to fluctuations and show high mortality rates at salinities much higher or lower than their optimal concentration of 25% seawater; 100% seawater is 32 ppt and approximately 1000 mOsms (Knowlton and Kirby, 1984). Therefore, the transition from stenohaline to euryhaline must occur after larval life stages when regulatory mechanisms have developed that provide sufficient osmoregulatory capacity under osmotic stress. As the embryonic period in grass shrimp comes before the larval period of development, they too must have limited range of salinity tolerance. The ability to withstand fluctuations in salinity must be largely due to the physical characteristics of the chorion acting as a physical barrier to environmental salinity stress.

The chorion of the grass shrimp has been previously characterized as a dynamic structure that is able to form additional layers throughout embryonic development period (Glas *et al*., 1997). The initial embryonic coat is produced within hours of fertilization and remains until 7

days post-fertilization when multiple embryonic envelopes become incorporated within the chorion and persist until eclosion. In the grass shrimp, it is approximately 8 days post-fertilization when the heart begins to contract at stage VIb (Romney and Reiber, submitted to *Crustaceana*, 2011). The underdeveloped chorion at this stage may allow water movements between the animal and its environment. The result of this might induce the initiation of cardiac activity that likely occurs in response to hemolymph volume changes with the embryo under osmotic stress. When cardiac activity is initiated at stage VIb, contractions are arrhythmic and are likely less effective in the coordinated movement of hemolymph and thus not optimal for gas exchange and maintaining metabolism. In addition, the individual parameters that make up V_b such as f_H and V_s are higher at 45 ppt salinity as compared to control conditions of 30 ppt. As the cardiovascular system matures (with embryonic development), the rate of increase of V_b from stage VIb to VIII appears to be greater at 45 ppt than compared to control embryos at 30 ppt.

In summary, exposure to large fluctuations in environmental salinity occurs often during the embryonic development of grass shrimp as the fertilized eggs are transported externally on maternal pleopods. As larvae, the shrimp can gain the ability to tolerate salinity stress as they develop the osmoregulatory mechanisms essential to maintain internal hemolymph osmotic pressures. During the embryonic period, we have shown here that exposure to hypersaline water can induce the embryos to lose water osmotically. Embryos can then compensate for hemolymph volume loss by increasing f_H and decreasing V_s to maintain optimal levels of V_b and EF that are necessary for convective transport. Future work to evaluate internal hemolymph hydrostatic and osmotic pressure changes in response to salinity fluctuations would provide further insight into the regulatory mechanisms that exist with the embryonic shrimp heart. By understanding fundamental physiological responses to salinity, the field of developmental biology can better understand the effects of environmental fluctuation, like global climate change, on early life stages in animals.

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CHAPTER 5

ONTOGENY AND CARDIAC PHYSIOLOGY OF THE EMBRYONIC GRASS SHRIMP, *Palaemonetes pugio*

RESEARCH CONCLUSIONS

Investigations of crustacean physiological development aim to understand the fundamental mechanisms of regulation that contribute to adult environmental tolerance (Anger, 2006; Burggren and Warburton, 2005; Spicer and Burggren, 2003). Such mechanisms are observed as a response to environmental stress and thus can be easily incorporated into laboratory experimental designs (Burggren and Warburton, 2005; Burggren and Pinder, 1991). Furthermore, invertebrate systems hold fundamental clues to the evolutionarily conserved regulatory systems that exist in many advanced forms of cardiac function (Burggren and Pinder, 1991; McMahon *et al*., 1997).

Decapod shrimp can tolerate large fluctuations in environmental parameters such as temperature, salinity, and dissolved oxygen (Anderson, 1985; Morgan, 1980; Roesljadi *et al*., 1976; Thorp and Hoss, 1975; Welsh, 1975; Wood, 1967). They are able to successfully survive and reproduce in these environments that often fluctuate in salinities between 0 to 55 parts per thousand (seawater at 30-32 parts per thousand; ppt). The family Palaemonidae is considered by some to be in the process of evolution from marine to fresh water (Emery K. O. *et al*., 1967; Knowlton and Kirby, 1984). This would be particularly advantageous for their survival considering their inhabiting brackish coastal waters. Any observed distribution patterns of adult *Palaemonetes* spp. in relation to salinity may be accounted for by their relative ability to tolerate a particular range of salinity (and extent of change over this range) and their ability to maintain

internal water volumes and cardiac performance. However, many of these homeostatic mechanisms do exist in early life stages such as embryonic and larval development.

Ontogeny can be described for many decapod crustaceans as a complex transition both morphologically and physiologically in a series of gradual changes within a life cycle (Carlson, 2003; Reiber and Harper, 2001). Regular development is defined by Broad (1957) and reiterated by Gore (1985), as the pattern of development (in terms of numbers of stages) that predominates in decapods. We have demonstrated here that *P. pugio* shows an anamorphic pattern as a larva that is frequently interrupted by a molt stage that shows a radical change in morphology and/or physiology, a metamorphosis. It is believed that having multiple developmental stages might allow a wider degree of plasticity in such morphological/physiological parameters that are necessary for animals whose habitats are characteristic of fluctuating environmental variables (Reiber and Harper, 2001).

From the studies conducted here, the embryonic grass shrimp, *Palaemonetes pugio*, appears to offer the possibility that cardiac compensatory responses may exist during embryonic development to facilitate optimal cardiac function. The grass shrimp were able to adjust cardiac parameters such as heart rate and stroke volume as compensatory mechanisms in order to withstand internal water loss due to salinity stress. In hypersaline water, embryonic hemolymph volume is subject to osmotic influences at the chorion that would otherwise dehydrate the developing embryo due to the lack of their osmoregulatory capability. Embryos exhibited cardiac responses in order to compensate for hemolymph composition changes and maintain levels of cardiac output and ejection fraction that is likely necessary for the developing tissues.

Much of what we know regarding crustacean cardiovascular development comes from physiological and regulatory observations in response to environmentally induced stress (Anger, 2003; Burggren, 1988; Guadagnoli and Reiber, 2005; Harper and Reiber, 2004; McGaw and

McMahon, 1996; McGaw and Reiber, 1998; McGaw and McMahon, 2003; Reiber, 1997; Reiber and Harper, 2001; Wilkens, 1999). The advent of internal convective processes early in development and during their responses to high salinity serve to increase oxygen transport rates thus allowing *P. pugio* to partially compensate for embryonic water loss. Cardiac compensatory reflexes resulting from hemolymph volume changes have been demonstrated in adult decapods (Burggren *et al*., 1990; McGaw and Reiber, 1998). This study along with other investigations of physiological ontogeny in crustaceans provides a unique opportunity to better understand processes of cardiac malleability and physiological trajectories. Here we have shown that significant differences in cardiac performance, as quantified by stroke volume and ejection fraction at high salinities as an induced phenotypic response from the environment.

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Thesis Title: Embryonic Development of the Grass Shrimp, *Palaemonetes pugio*, and the Influence of Salinity on Cardiac Physiology

Thesis Examination Committee:

Chairperson, Carl L. Reiber, Ph. D
Committee Member, Michelle M. Elekonich, Ph. D
Committee Member, Stephen P. Roberts, Ph. D
Graduate Faculty Representative, John Young, Ph. D