

Physiological Development of the Embryonic and Larval Crayfish Heart

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Abstract. The cardiovascular system is the first system to become functional in a developing animal and must perform key physiological functions even as it develops and grows. The ontogeny of cardiac physiology was studied throughout embryonic and larval developmental stages in the red swamp crayfish *Procambarus clarkii* using videomicroscopic dimensional analysis. The heart begins to contract by day 13 of development (at 25 °C, 20 kPa O₂). Cardiac output is primarily regulated by changes in heart rate because stroke volume remains relatively constant throughout embryogenesis. Prior to eclosion, heart rate and cardiac output decreased significantly. Previous data suggest that the decrease in cardiac parameters prior to hatching may be due to an oxygen limitation to the embryo. Throughout development, metabolizing mass and embryonic oxygen consumption increased, while egg surface area remained constant. The surface area of the egg membrane is a constraint on gas exchange; this limitation, in combination with the increasing oxygen demand of the embryo, results in an inadequate diffusive supply of oxygen to developing tissues. To determine if the decrease in cardiac function was the result of an internal hypoxia experienced during late embryonic development, early and late-stage embryos were exposed to hyperoxic water (P_{O₂} = 40 kPa O₂). Heart rate in late-stage embryos exposed to hyperoxic water increased significantly over control values, which suggests that the suppression in cardiac function observed in late-stage embryos is due to a limited oxygen supply.

Introduction

Crustaceans exhibit a diverse array of metabolic and physiological responses to aquatic hypoxia (Reiber, 1995). Typically, hypoxic exposure results in a decreased heart rate (bradycardia) in decapod crustaceans (McMahon and Burnett, 1990; Wilkens, 1993; McGaw *et al.*, 1994; Reiber, 1995; Reiber and McMahon, 1998). This hypoxia-induced bradycardia has been well documented in adult red swamp crayfish (*Procambarus clarkii*) (Reiber, 1995, 1997; Chapman and Reiber, 1998; Reiber and McMahon, 1998). However, an examination of heart rate alone does not provide a complete picture of cardiac performance. Cardiac output and stroke volume are broader measures of cardiac performance that can vary over wide ranges with little or no variation in heart rate (Orlando and Pinder, 1995). Furthermore, heart rate and stroke volume can vary independently of each other in decapods (McMahon and Burnett, 1990). Therefore, the cardiovascular response of decapods to a given perturbation can be understood only by evaluating heart rate, stroke volume, and cardiac output rather than just heart rate.

Cardiac performance in adult red swamp crayfish is altered when the animals are exposed to levels of oxygen below the critical level needed to maintain aerobic metabolism (P_{CRIT}) (approximately 5 kPa O₂ at 25 °C) (Reiber, 1995). Reiber (1995) found a decrease in heart rate and an increase in stroke volume in adult red swamp crayfish exposed to hypoxic conditions. The increase in stroke volume and maintenance of cardiac output is likely due to increased pericardial sinus pressure coupled with increased filling pressure, an increase in diastolic filling time, and an increased end diastolic volume (Reiber, 1995).

Information on cardiac function and hypoxic responses in embryonic and larval crustaceans is limited (Spicer, 1994;

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Spicer and Morritt, 1996; Reiber, 1997). Cardiac functions in embryos and larvae can be quite different from those of adults because centers of metabolic activity shift during development (Reiber, 1997). Additionally, embryonic crayfish cannot escape hypoxic waters: the embryos are attached to the female's pleopods, and the brooding female typically remains sequestered within the burrow where the water can become oxygen-depleted due to both crayfish and microbial respiration (Payette and McGaw, 2003). To survive, the embryonic and larval crayfish must possess physiological mechanisms for dealing with hypoxia, and these may differ from adult responses. Fluid convection on either side of the egg membrane would allow for greater oxygen extraction from the environment by eliminating boundary layers and maintaining a diffusional gradient. The embryo itself does not move around within the egg to aid convective processes, so cardiac development is critical to embryonic viability because it provides the only source of internal convection to facilitate gas movement within the egg (Seymour and Bradford, 1995).

In late-stage crayfish embryos, E-Stage XVI, cardiac activity is not significantly responsive to hypoxic exposure, whereas in E-Stage XIV, the heart rate decreases significantly when water P_{O_2} is dropped to 5 kPa (an adult-like response) (Reiber, 1995; refer to table 1 for staging). Reiber (1995) attributed the lack of response in the late-stage embryos to the possibility that they are already internally hypoxic (experiencing levels of oxygen below their metabolic demands). This hypothesis was supported by the finding that the heart rates for animals at E-Stage XVI were the same as those for E-Stage XIV embryos during hypoxic exposure (2 kPa) (Reiber, 1995).

The current study evaluates cardiac physiology and metabolic function throughout early development of the red swamp crayfish. To determine whether embryos are experiencing an internal hypoxia just prior to hatching, oxygen consumption was measured along with metabolizing mass and exchange surface area throughout development. Subsequently, early and late-stage embryos were exposed to acutely hyperoxic conditions. It was expected that cardiac function in late stage embryos would be impaired due to an internal hypoxia caused by an increase in metabolizing mass and the limits in the surface area for gas exchange. Such physiological cardiovascular impairment would result in a decrease in cardiac parameters that would be alleviated by exposure to hyperoxic water.

Materials and Methods

Crayfish breeding

Adult male and female crayfish (*Procambarus clarkii* [Girard, 1852]) were purchased from the Atchafalaya Biological Supply Co., Inc. Animals were maintained in the laboratory in 35-l aquaria (5–6 animals to an aquarium)

filled with dechlorinated tap water (25 °C, pH = 7.0, conductivity = 150–300 μ S) and fed twice a week (romaine lettuce and liver). Mature females were separated from other animals and placed into individual 2-l containers (25 °C and 10:14 dark/light cycle), where they were held for 3–4 days, after which a sexually mature male was placed in the container and the pair was observed for mating behavior. If mating did not ensue within 20 min, the male was replaced with another male and again observed for copulatory behavior. If copulation occurred, the animals were left together for 5 h and then separated to prevent cannibalism. After mating, females were placed into a nursery aquarium (25 °C) and observed daily until eggs were laid.

Experimental apparatus

Crayfish embryos or larvae were removed from females, staged according to Harper and Reiber (2001), and then attached to an applicator stick using cyanoacrylate gel glue. A minimal amount of gel glue was applied to the flattened end of the applicator stick and pressed against the egg or larva. Care was taken to minimize obstruction of the egg's respiratory surface area yet provide a firm holdfast for attachment. Animals were then placed into a flow-through experimental chamber (Harper and Reiber, 1999) where they were held for 30 min. Larval crayfish were not observed to struggle or show swimming behaviors during the experimental period. Water (25 °C, pH = 7.0, P_{O_2} = 20 kPa) was pumped (12 ml \cdot min⁻¹) through the experimental chamber and then over a Clark-type polarographic oxygen electrode to determine P_{O_2} . The P_{O_2} and temperature of the reservoir water were established and maintained using a gas mixing system (Cameron Instrument Company, GF-3/MP) and a circulating water bath (VWR Scientific, 1160). Changes in heart shape were monitored using a microscope (Leica MZ 12.5) equipped with a video camera (Mintron, 05-70D), super VHS video recording system (Panasonic, PV-4661), and Horita time code generator (VG-50).

Ontogeny of heart rate, stroke volume, and cardiac output

Seven crayfish from each of seven developmental stages were used: embryonic stages E-Stage XIII, E-Stage XIV, E-Stage XV, and E-Stage XVI, and larval stages L-Stage I, L-Stage II, and L-Stage III (see Reiber, 1995; and Reiber and Harper, 2001, for details on the correlation between days of development at 25 °C and developmental stage for these stages). Heart rate and stroke volume were obtained using frame-by-frame (60 Hz sampling speed) analysis of the videotape on an editing tape player (Panasonic, AG-DS550). Heart rate for each animal is presented as the mean number of beats per minute calculated from three 30-s intervals. To determine cardiac volumes (end diastolic volume and end systolic volume), embryonic and larval crayfish hearts were modeled as a prolate spheroid (cardiac

volume = $4/3\pi ab^2$), where a and b are half of the measured long and short axes of the heart, respectively (Keller *et al.*, 1991, 1994; Taber *et al.*, 1992; Schwerte and Pelster, 2000; and Harper and Reiber, 2001). In early embryos (Stage XIII), the length of both the long and short axes of the heart were averaged over a minimum of 10 cardiac cycles to account for peristaltic-like contractions when the heartbeat is initiated. The geometric equation used to model the heart takes into account cardiac growth with development (*i.e.*, the increasing length and width of the heart as measured within each developmental stage). In later developmental stages (embryonic and larval) the heart remains a prolate spheroid; it changes only in its ratio of length to width. The equation for a prolate spheroid can be applied even if the changes are somewhat disproportional (*i.e.*, a longer thinner prolate spheroid models mathematically the same as a shorter thicker prolate spheroid). The basic geometry of the heart remains the same throughout development, with changes occurring primarily in the long and short axes. Stroke volume was calculated from the difference between end diastolic volume (EDV) and end systolic volume (ESV). Cardiac output was calculated as the product of stroke volume and heart rate. Heart rate, stroke volume, and cardiac output were determined for each developmental stage.

Oxygen consumption

Closed-system respirometry was used to determine embryonic oxygen consumption. Six animals were placed in a 50-ml syringe filled with aerated water and placed on an oscillating plate at 25 °C. A 5-ml water sample was injected into a chamber holding a Clark-type polarographic oxygen electrode after 30, 60, and 90 min. Three replicates were run for each of the following stages: E-Stage II, E-Stage XIII, E-Stage XV, E-Stage XVI, L-Stage I, and L-Stage III. Two syringes lacking animals were run to account for bacterial respiration, which was found to be negligible. Fluid volume and water P_{O_2} in the experimental chamber were decreased by a total of 15 ml and 6.6 kPa O_2 (minimum) over the 90-min course of the experiments. This change in volume was adjusted for when calculating oxygen consumption. Rates of oxygen consumption over the experimental period were consistent, indicating that the small reduction in oxygen availability did not adversely affect the animals. Mass-specific oxygen consumption is reported as microliters of O_2 per milligram (wet mass) per hour. Oxygen consumption rate was calculated according to the formula $VO_2 = (V_r \times \Delta P_{WO_2} \times \beta_{WO_2}) / \Delta t \times W$; where VO_2 is oxygen consumption, V_r is the volume of water in the respirometer, ΔP_{WO_2} is the change in water P_{O_2} , β_{WO_2} is the capacitance of oxygen in water, Δt is the duration in minutes, and W is the wet weight of the animal.

Lipid and metabolizing mass

Embryonic crayfish were collected from gravid females at E-Stage II, E-Stage XIII, E-Stage XV, and E-Stage XVI, and larval crayfish were collected at L-Stage I and L-Stage III. Egg diameter and larval length were measured ($n = 7$ per stage). Egg surface area was calculated using the formula for a sphere (surface area (mm^2) = $4\pi r^2$). Surface area was calculated only for embryos. Each embryo and larva was dried with a Kimwipe for 10 s prior to weighing and placed on a clean, preweighed coverslip. To determine the metabolically active mass (tissue) of the embryo or larva, the nonmetabolic (lipid) portion of the body was first removed by puncturing the wall of the animal and isolating the lipid onto the coverslip. The lipid-free animal was then weighed. The egg membrane and exoskeleton were not separated from the active metabolizing mass but were assumed to contribute little to mass error because they constitute only a small proportion of overall mass. Furthermore, errors in this estimate were likely similar among stages of development. Non-lipid mass was calculated as the difference in animal mass before and after removal of lipids.

Hyperoxic exposure

Animals at E-Stage XIV ($n = 13$) and E-Stage XVI ($n = 13$) were held individually under experimental chamber conditions for 30 min followed by heart rate measurements to determine control values. Control and experimental P_{O_2} values were established and maintained using a gas mixing system (Cameron Instrument Company, GF-3/MP). Embryos were exposed to normoxic (20 kPa O_2) and hyperoxic (40 kPa O_2) water for 30 min. Heart rate was determined as previously described for both control and experimental conditions at E-Stage XIV and E-Stage XVI.

Statistical analysis

Means and standard errors were calculated for each stage ($n = 7$) for data on egg surface area, lipid mass, metabolizing mass, and oxygen consumption rate. An analysis of variance was used to determine overall effects of development on the dependent variables (SigmaStat, ver. 2.03). A Bonferroni t -test was used for pairwise multiple comparisons where a significance of $P \leq 0.05$ was found. Lipid/metabolizing mass ratios failed normality tests, thus an analysis of variance based on ranks and a Tukey test were used. Baseline cardiac parameters were established by pooling values obtained for each stage ($n = 7$) and calculating a mean and standard error. The effects of developmental stage on cardiac parameters were assessed using a one-way analysis of variance (SigmaStat, ver. 2.03). Where developmental effects were shown to be significant ($P < 0.05$), group means were compared using the Newman-Keuls multiple range test. A Student's t -test was used to compare

embryonic response to normoxic and hyperoxic exposure with significance at the level of $P = 0.05$. Embryos within a developmental stage ($n = 13$) were used for comparison.

Results

Ontogeny of heart rate, stroke volume, and cardiac output

Uncoordinated cardiac contractions began in embryonic crayfish by E-Stage XIII of development, with a mean heart rate of 160 ± 3 (SEM) beats \cdot min $^{-1}$ (bpm). At the onset of cardiac contractions heart rate was irregular, characterized by short bursts of activity (e.g., 300 bpm), very long peristaltic-like contractions (e.g., 48 bpm), and intermittent periods of cardiac arrest (maximum of 11 s). Therefore, heart rate was determined by averaging over the six 30-s time periods. Heart rate varied significantly with development ($P = 0.05$, $F = 7.29$). Mean heart rate remained unchanged (163 ± 2 bpm) through E-Stage XIV, after which it increased significantly ($P = 0.05$, $F = 3.64$) to 192 ± 6 bpm at E-Stage XV. At E-Stage XVI, heart rate decreased significantly ($P = 0.05$, $F = 3.01$) to 149 ± 3 bpm and remained at this rate until eclosion. Upon hatching, heart rate increased significantly ($P = 0.05$, $F = 5.11$) to 255 ± 9 bpm and remained at this level through all three larval stages (Fig. 1a).

Both stroke volume and cardiac output showed little variation throughout embryonic development (Fig. 1b, c). However, after hatching (L-Stage I), stroke volume increased significantly ($P = 0.05$, $F = 42.17$) from 3.8 ± 0.9 (SEM) nl/beat to 12.4 ± 1.8 nl/beat, and cardiac output increased significantly ($P = 0.05$, $F = 281.55$) from 178 ± 96 (SEM) nl \cdot min $^{-1}$ pre-hatching (E-Stage XVI) to 3130 ± 46 nl \cdot min $^{-1}$ at the first larval stage. Stroke volume decreased significantly ($P < 0.001$, $F = 21.64$) from the first larval stage (12.4 ± 1.8 nl \cdot beat $^{-1}$) to the second larval stage (6.07 ± 1.9 nl \cdot beat $^{-1}$), with no significant change occurring from the second to third larval stages. During larval development, cardiac output followed stroke volume, decreasing significantly ($P = 0.05$, $F = 77.17$) at L-Stage II and then remaining stable into L-Stage III (1660 ± 43 to 1747 ± 47 nl \cdot min $^{-1}$).

The effects of cardiac differentiation and elongation on the ontogeny of cardiac physiology can be assessed using the data relating the length and width of the heart at end diastole to days of development (Fig. 2). The pattern of cardiac development reveals that the heart grows in length from 8.3 ± 0.3 (SEM) to 11.0 ± 0.4 μ m (32.5% increase) as compared to width from 5.9 ± 0.2 (SEM) to 8.4 ± 0.3 μ m (42.4% increase), through embryonic development. There is a greater absolute increase in the length as opposed to the width of the heart during development, which results in a dramatic increase in the length-to-width ratio after eclosion.

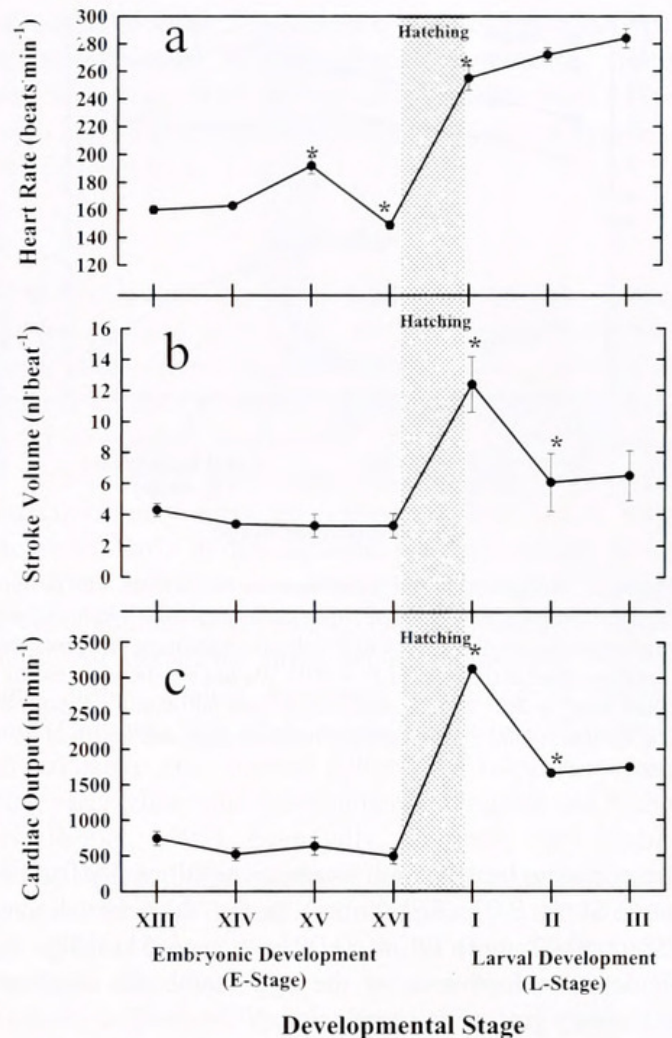


Figure 1. Ontogeny of (a) heart rate, (b) stroke volume, and (c) cardiac output in the red swamp crayfish *Procambarus clarkii*. * indicates significant difference from the previous stage at the level of $P = 0.05$. Shaded area indicates when hatching occurs. Values are shown as means \pm standard error, $n \geq 7$ at each stage. Embryonic and larval stages correspond to the following days of development at 25 $^{\circ}$ C: E-Stage XIII = 12–14 days, E-Stage XIV = 14–17 days, E-Stage XV = 17–19 days, E-Stage XVI = 19–21 days, L-Stage I = 21–23 days, L-Stage II = 23–25 days, and L-Stage III = 25–27 days.

Oxygen consumption

Oxygen consumption increased significantly between E-Stage IV (4.03 ± 0.50 (SEM) μ l $O_2 \cdot$ mg $^{-1} \cdot$ h $^{-1}$) and E-Stage XIII (71.33 ± 5.19 μ l $O_2 \cdot$ mg $^{-1} \cdot$ h $^{-1}$) ($P = 0.05$, $t = 4.05$) and between E-Stage XIII and E-Stage XV (148.14 ± 13.61 μ l $O_2 \cdot$ mg $^{-1} \cdot$ h $^{-1}$) ($P = 0.05$, $t = 5.21$) (Fig. 3). Just before hatching, E-Stage XVI, metabolic oxygen consumption did not increase significantly from the previous stage. Larval stages showed a significant increase in oxygen consumption (L-Stage I, $P < 0.001$, $t = 7.597$ and L-Stage III, $P < 0.001$, $t = 16.953$).

Lipid/metabolizing mass

Embryo diameter (used to calculate membrane area) did not change significantly through embryonic development.

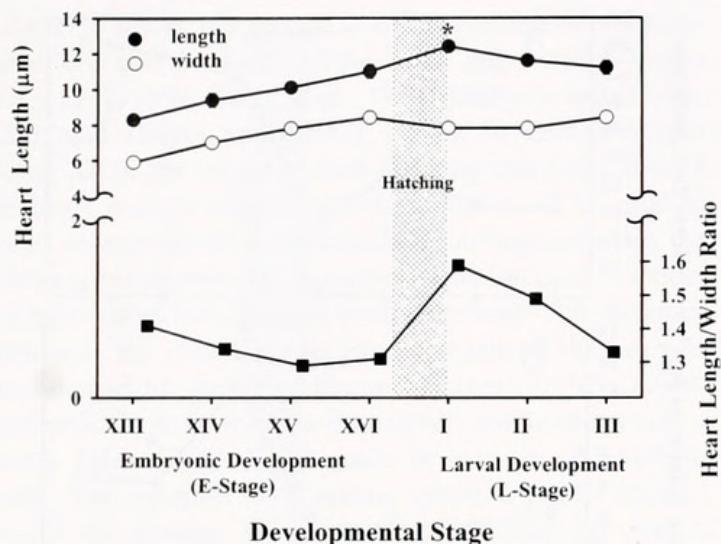


Figure 2. Length and width measurements of the heart with development. Solid circles represent length, open circles represent width, and solid squares represent length/width ratio. * indicates significant difference from the previous stage at the level of $P = 0.05$. Values are shown as means \pm standard error, $n \geq 7$ at each stage. Error bars fall within symbols. See legend to Figure 1 for correspondence between stage and length of development.

After eclosion, larval length increased significantly from the first (2.61 ± 0.03 (SEM) mm) to the third larval instar (3.38 ± 0.02 mm) ($P = 0.001$, $t = 5.61$) (Fig. 4a). Calculated surface area of the egg membrane decreased significantly just prior to eclosion ($P = 0.05$, $t = 3.17$) (Fig. 4b). Animal mass also decreased significantly just

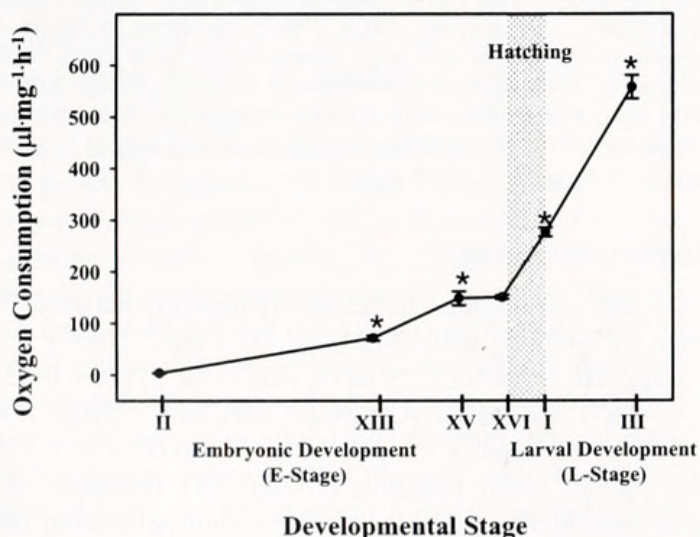


Figure 3. Oxygen consumption for embryonic and larval crayfish. * indicates significant difference from the previous stage at the level of $P = 0.05$. Shaded area indicates when hatching occurs. Values are shown as means \pm standard error, $n \geq 3$ at each stage. Embryonic and larval stages correspond to the following days of development at 25 °C: E-Stage II = 2–4 days, E-Stage XIII = 12–14 days, E-Stage XV = 17–19 days, E-Stage XVI = 19–21 days, L-Stage I = 21–23 days, and L-Stage III = 25–27 days.

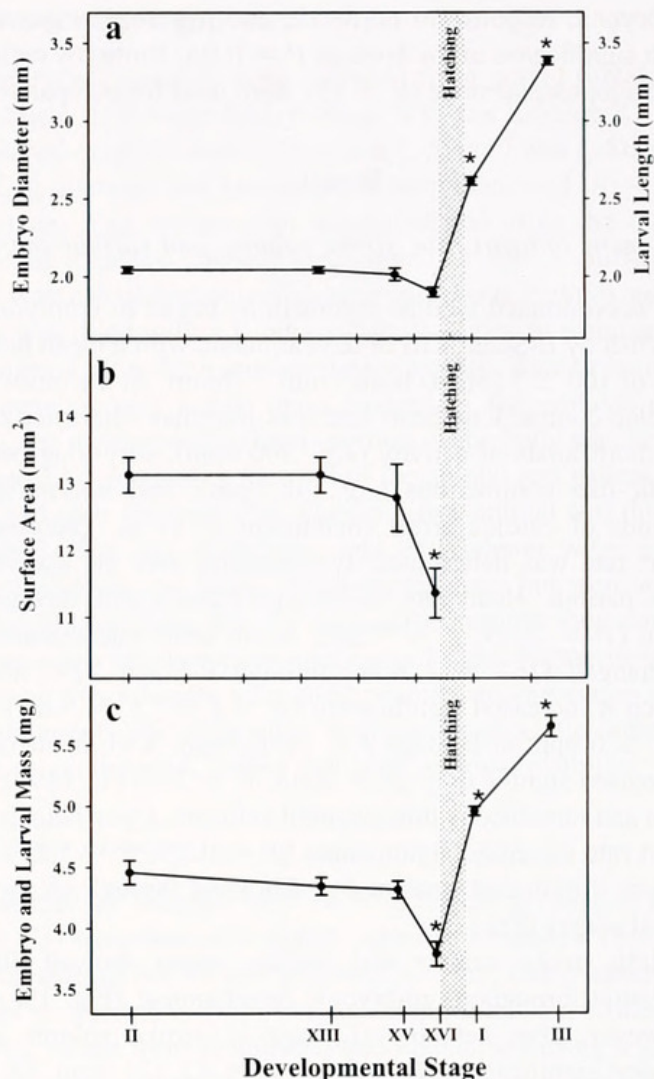


Figure 4. (a) Diameter of embryo or length of larval crayfish at various stages throughout development. (b) Surface area calculated from diameter measurements on embryonic crayfish. Surface area measurements not applicable to larvae. (c) Mass of developing crayfish. * indicates significant difference from the previous stage at the level of $P = 0.05$. Shaded area indicates when hatching occurs. Values are shown as means \pm standard error, $n \geq 7$ at each stage. See Figure 3 for correspondence between stage and length of development.

prior to hatching (4.31 ± 0.07 (SEM) mg to 3.78 ± 0.10 mg) ($P < 0.001$, $t = 4.65$) but increased significantly by the first larval stage (3.78 ± 0.10 mg to 4.95 ± 0.03 mg) ($P < 0.001$, $t = 10.31$) (Fig. 4c).

Lipid content of the egg decreased significantly from E-Stage XIII (2.41 ± 0.06 (SEM) mg) to E-Stage XV (1.85 ± 0.20 mg) ($P = 0.05$, $t = 3.52$) and from L-Stage I (1.64 ± 0.06 mg) to L-Stage III (0.64 ± 0.02 mg) ($P < 0.001$, $t = 6.32$) (Fig. 5a). As lipid content decreased, metabolizing mass increased significantly between E-Stage XIII (1.93 ± 0.08 mg) and E-Stage XV (2.46 ± 0.17 mg) ($P = 0.05$, $t = 3.92$) (Fig. 5b). Metabolizing mass increased throughout embryonic development, although egg membrane surface area did not change significantly. The metabolizing mass of the first larval stage (3.31 ± 0.04 mg)

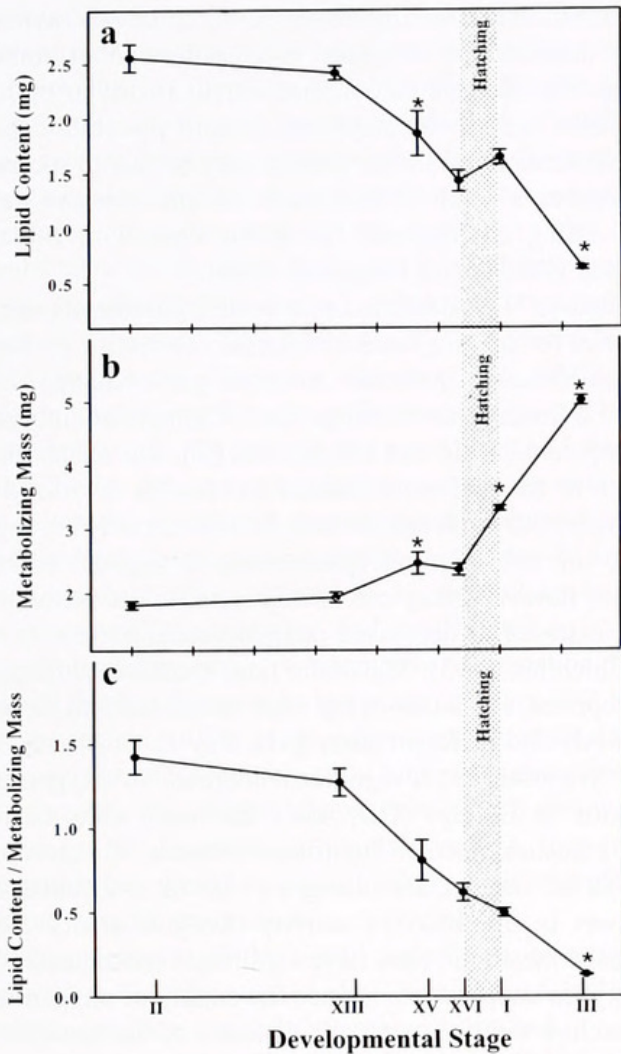


Figure 5. (a) Lipid content of developing crayfish. (b) Metabolizing mass of developing crayfish. (c) Ratio of lipid content to metabolizing mass in the developing crayfish. * indicates significant difference from the previous stage at the level of $P = 0.05$. Shaded area indicates when hatching occurs. Values are shown as means \pm standard error, $n \geq 7$ at each stage. See Figure 3 for correspondence between stage and length of development.

($P < 0.001$, $t = 6.35$) increased significantly by the third larval stage (5.01 ± 0.07 mg) ($P < 0.001$, $t = 12.598$). The ratio of lipid to metabolizing mass declined significantly ($P = 0.001$, $q = 7.040$) over the embryonic and larval development period from 1.43 ± 0.10 (SEM) at E-Stage IV to 0.12 ± 0.01 at L-Stage III but did not change significantly from one stage to the next (Fig. 5c).

Hyperoxic exposure

E-Stage XIV and E-Stage XVI embryos were selected to represent animals thought to be under internally normoxic and hypoxic conditions, respectively. If embryonic cardiac function is depressed in late-stage embryos because they are oxygen-deficient, then an increase in the oxygen diffusion gradient should partially restore oxygen delivery and thus allow heart rate to increase. No significant change was

observed in E-Stage XIV animals when exposed to hyperoxic water (Fig. 6). However, heart rate increased significantly in E-Stage XVI animals ($P < 0.001$, $F = 21.73$) (from 138 ± 6 to 159 ± 6 bpm) when exposed to hyperoxic water (40 kPa O_2).

Discussion

The mechanisms by which embryonic cardiac contractions are initiated in crayfish are not clearly understood. Cardiac contractions, which result in the movement of hemolymph within the embryo, appear to facilitate gas transport in developing crustaceans (Reiber, 1997). Burggren and Territo (1995) have suggested that these early cardiac contractions may serve an angiogenic function in lower vertebrates. Early in development, the fundamental driving forces that influence developing physiological systems are often similar, even among taxa from diverse animal groups. All organisms must balance immediate environmental needs against long-term physiological requirements. Crayfish, like other animals, rely on diffusion for both exchange and internal delivery of respiratory gases during early embryonic development (Burggren and Pinder, 1991; Reiber, 1997). Eventually, however, they attain a mass at which diffusion alone can no longer adequately meet gas exchange demands; at this point the active movement of fluids (hemolymph) as a result of cardiac pumping is initiated. The heart rate in most animals changes as development proceeds from embryo through immature or larval stages to adult, yet the patterns of change are not consistent among species in lower vertebrates (Burggren and Pinder, 1991; Farrell, 1991; Hou and Burggren, 1995; Icardo, 1996) or invertebrates (Cooke, 1988; Spicer and Morritt, 1996).

Spicer and Morritt (1996) have shown that the timing of

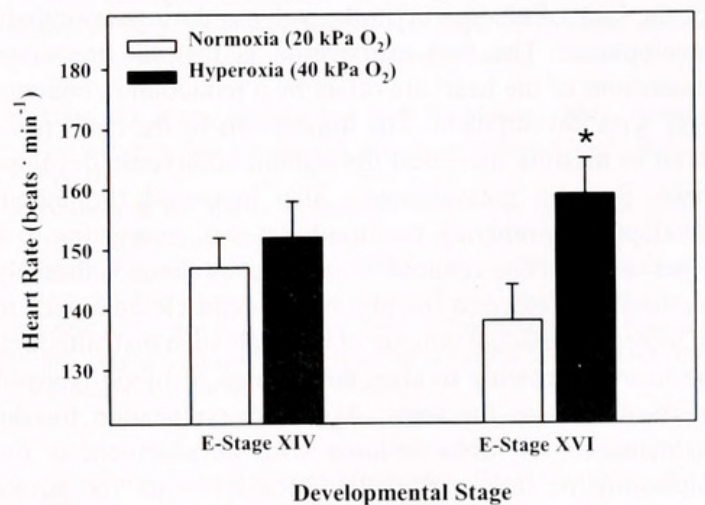


Figure 6. Heart rate of two stages of embryonic crayfish exposed to hyperoxic water (40 kPa). * indicates significant difference from control ($P = 0.05$). E-Stage XIV correlates to 14–17 days of development at 25 $^{\circ}$ C and E-Stage XVI to 19–21 days.

key developmental events, such as the initiation of cardiac contractions, is not consistent among aquatic crustaceans. However, some generalizations can be made about the ontogeny of cardiac physiology and function from the first appearance of cardiac contractions to maturity. Whether segmentation in an animal occurs pre- or post-hatch appears to be linked with the onset of beating of the heart because segmentation of the thoracic cavity precedes initiation of the heartbeat (Spicer, 1994; Spicer and Morritt, 1996). The relationship between body mass and heart rate does not conform to a single power curve model; rather it depends on the organogenesis of the heart itself. In the crayfish *Procambarus clarkii*, the heart grew proportionally in width and length throughout embryonic development; yet just after hatching, the length of the heart increased in slightly greater proportion than width, resulting in a large and rapid increase in the length-to-width ratio. As suggested by Spicer and Morritt (1996), this probably represents the switch from differentiation of cardiac tissue to the elongation phase of cardiac development. After hatching, larval mass increased significantly while heart rate remained fairly constant. This developmental pattern—a decrease in heart rate with increasing body size after cardiac development is complete and elongation has commenced—was described by Spicer and Morritt (1996) in the water flea *Daphnia magna*, the amphipod *Gammarus duebeni*, the lobster *Nephrops norvegicus*, and the brine shrimp *Artemia franciscana*.

Many adult crustaceans adjust stroke volume rather than heart rate to modulate cardiac output (Reviewed by McMahon and Burnett, 1990; McGaw *et al.*, 1994; Reiber, 1995; Reiber and McMahon, 1998). However, stroke volume does not appear to be as tightly regulated as heart rate during the embryonic development of crayfish. Embryonic crayfish apparently use heart rate as a primary mechanism to regulate cardiac output, as stroke volume remains constant throughout embryogenesis. We present three explanations for the lack of change in stroke volume during embryonic development. The first explanation is that the increased dimensions of the heart are offset by a reduction in contractility with development. The dimensions of the heart measured in diastole increased throughout embryonic development. Systolic measurements also increased throughout development, implying that the heart was contracting to a lesser degree. The reduced force of contraction ultimately decreases the ejection fraction of the ventricle and leads to an increased residual volume of the heart such that, although the heart is growing in size, the volume of blood pumped per beat remains the same. A second explanation for the maintenance of stroke volume with development is the thickening of the heart walls. Measurements for stroke volume were obtained using the outer diameter of the heart, which may not account for thickening of the myocardium with development. After hatching, elongation of the heart and a decrease in systolic volume contribute to the signifi-

cant rise in stroke volume observed in the larval crayfish. It is the maintenance of stroke volume throughout embryogenesis that dictates that cardiac output is driven by heart rate alone early in development. A third possibility is that the maintenance of stroke volume may be due to increased afterload as a result of increased vascular pressure developed during angiogenesis, as is the case with zebrafish embryos (Pelster and Burggren, 1996).

Reiber (1997) postulated that cardiac parameters decline just prior to hatching because internal convective processes are insufficient to facilitate adequate gas exchange in the face of a limited gas exchange area. Throughout embryonic development in the red swamp crayfish, the metabolizing portion of the embryo increased in mass as it utilized the lipid-rich yolk as metabolic fuel. Embryonic crayfish appear to rely on oxidative phosphorylation throughout embryogenesis; however, they can switch to anaerobic metabolism when exposed to decreased oxygen concentrations (Chiba and Chichibu, 1993). Metabolic rates increased throughout development as metabolizing mass increased and as organogenesis and differentiation gave way to functional systems. We observed a significant increase in oxygen consumption at E-Stage XIII, when the heart starts beating. After eclosion, another significant increase is observed in each larval stage; these changes could be associated with increases in the animal's activity (DeSilva *et al.*, 1986). Increased metabolic rates have significant consequences for the oxygen supply system since the supply of oxygen must be matched with the metabolic demands of the embryo. We observed an increase in the oxygen consumption of the crayfish throughout embryonic and larval development, except at the point prior to hatching when the embryos are thought to be internally hypoxic and could be relying on anaerobic metabolic pathways.

Embryonic aerobic metabolism cannot be sustained under severe hypoxic conditions. Myocardial anaerobic capabilities are typically limited or nonexistent, which makes this tissue particularly sensitive to hypoxia; thus, if the embryo can no longer maintain aerobic metabolism, the heart should already be failing. Cardiac function declined during embryonic development in the crayfish, most likely due to an internal hypoxia resulting from the embryo's oxygen requirements exceeding the diffusive capacity of its outer egg membranes. Prior to hatching, an internal hypoxia could result in direct inhibition of cardiac metabolism and cardiac function, as is observed in larval frogs (Fritsche and Burggren, 1996). In the crayfish, eclosion removes the physical limitation to gas exchange (the egg membrane) and active respiratory mechanisms (gill ventilation) are initiated. Cardiac functions can then increase after hatching, as the animal becomes more active and organ systems mature.

To determine whether the diminution in cardiac function observed in late-stage embryos was due to an internal hypoxia, the diffusional gradient of oxygen was increased by

exposing the embryo to hyperoxic water. The finding that this exposure increased the heart rate in late-stage crayfish embryos but not in early embryos suggests that oxygen limitation is at least one factor responsible for reducing the heart rate in late-stage embryos. However, the increase in heart rate observed in late-stage embryos exposed to hyperoxia could also be explained by changes in the oxygen conductance of the egg membrane. In amphibian eggs, the oxygen conductance of the egg membrane is related to the stage of the embryo and is not directly influenced by environmental factors (Seymour and Bradford, 1995). If the same is true for crayfish, the early stages might not respond to an increase in PO_2 because the conductance of the membrane limits the oxygen diffusion across the membrane. However, this would not account for the reduction in heart rate observed in late-stage embryos. It is far more likely that these embryos are experiencing an internal hypoxia that decreases cardiac parameters and is alleviated by exposure to high ambient PO_2 . An alternative explanation for depressed cardiac function in late-stage embryos is a shift in the oxygen sensitivity of the embryos throughout development. It is possible that late-stage embryos are less sensitive to water PO_2 than earlier stages. However, if this were the reason for the depression of cardiac function, then increasing water PO_2 would not have had the observed effect on cardiac parameters in the late-stage embryos.

Other mechanisms that could underlie the observed patterns of cardiac function during development include (1) intrinsic changes in membrane permeability and in myocyte characteristics such as the ion channels, and (2) the development of, or changes in, extrinsic controls (Fritsche, 1997). The decreases observed in egg surface area and animal mass may contribute to the decreases observed in cardiac parameters just prior to hatching. Lastly, it should be noted that the shifts in heart rate and stroke volume could coincide with the pacemaker of the heart switching from a myogenic to a neurogenic mechanism of cardiac regulation (Yamagishi, 1990; Yamagishi and Hirose, 1992; Chapman and Reiber, 1998; Harper and Reiber, 2000). Previous data on the extrinsic regulatory mechanisms of the embryonic crayfish cardiovascular system have been difficult to interpret. Embryonic crayfish hearts appear to be initially myogenic and to become neurogenic later in development (Chapman and Reiber, 1998; Harper and Reiber, 2000). The timing of this event could correspond with the shifts in cardiac function observed during embryonic development in the crayfish.

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