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Anoxia tolerance, anaerobic metabolism, and the lack of a mitochondrial permeability transition in the ghost shrimp, Lepidophthalmus louisianensis, Schmitt, 1935

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A Thesis

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The Department of Biological Sciences

by

Jeremy Dale Holman
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DEDICATION

I would like to dedicate this thesis to all of my family and friends who have supported me emotionally and financially during this long journey. There are too many individuals to list here, but a few of the more critical ones include my recently deceased grandpa Arlie Holman, my grandmother Bobbie, my parental saviors Victor and Wanda Holman, my future wife Evelyn Tan, her mother Sucy Tan, my uncle Rodney Holman, my good friend and role model Joe Kesterson, my undergraduate mentors Drs. Warren Burggren, Louis Burnett, and Pamela Padilla, and Judy Morris and Dr. Diana Elrod of the Ronald E. McNair program at the University of North Texas. I also dedicate this accomplishment to my country and to God.
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ABSTRACT

The ghost shrimp, *Lepidophthalmus louisianensis*, burrows up to meters deep in oxygen-limited marine sediments along the Gulf coast. During low tides these animals are subjected to extended periods of anoxia. The main objective of this study was to assess survival under anoxia and evaluate the physiological mechanisms that underlie the anoxia tolerance of this species.

I observed large specimens of *L. louisianensis* (>2g) having an LT<sub>50</sub> of 64 h under anoxia at 25º C. Smaller specimens (<1g) have a significantly higher LT<sub>50</sub> of 113 h under identical conditions (P<0.0001). I measured whole body lactate levels in shrimp exposed to anoxia for up to 72 h, and recorded significant accumulation of this anaerobic end product (ANOVA, P<0.001). I also measured adenylates and arginine phosphate in shrimp exposed to anoxia for up to 48 h, and after a 24-h recovery period. Adenylates were not significantly altered during the anoxia regime, and reductions in arginine phosphate occurred after 12 and 24 h, but returned to normoxic values during recovery (ANOVA, P<0.001). While reserves of arginine phosphate are used to some extent to buffer losses in ATP, substantial contribution to the maintenance of energetic status comes from the high rate of anaerobic glycolysis.

Energized mitochondria isolated from ghost shrimp hepatopancreas possess a pronounced ability to take up exogenous Ca<sup>2+</sup> (compared to mitochondria-free controls) as measured by following the external free Ca<sup>2+</sup> concentration with the fluorogenic dye Fluo-5N. Importantly, Ca<sup>2+</sup> was not released from the mitochondrial matrix at any level of exogenous Ca<sup>2+</sup> tested (up to 1.0 mM, in the presence of 5 mM phosphate). Thus, Ca<sup>2+</sup> does not stimulate opening of the mitochondrial permeability transition pore, which potentially could help prevent apoptotic and necrotic cell death during extended periods of anoxia. (Supported by NIH grant 1-RO1-GM0-71345-01 and by SIGMA XI GIAR).
INTRODUCTION

The ghost shrimp, *Lepidophthalmus louisianensis* (formerly *Callianassa jamaicense*) is a burrowing decapod crustacean of the infraorder Thalassinidea (Manning and Felder, 1991) that is commonly found in estuaries along the northern Gulf of Mexico. Dense populations of *L. louisianensis* are found in low-energy beaches, back-beach ponds, estuarine tidal flats, and tidal streams wherein they construct permanent and semi-permanent galleries up to several meters deep into the anoxic, sulfide-rich sediment (Willis, 1942; Britton and Morton, 1989; Felder, 1978; Felder and Griffis, 1994; Felder, 2001). These shrimp irrigate their burrows during high tides to replenish oxygen, flush out excretory waste, and oxidize sulfide diffusing into burrows from the sediment (Hagerman, 1998). When the tide recedes, the burrow water can become severely hypoxic or anoxic within hours, and during peak low tides or storm surges, these hypoxic conditions can last for days (Felder, 1979). As with all shallow bodies of water, the overlying water at high tide may become hypoxic at night, potentially adding to the duration that ghost shrimps must cope with extremely low ambient \( \text{PO}_2 \). *L. louisianensis*, among other species, have demonstrated a remarkable ability to withstand laboratory conditions mimicking severe hypoxia and anoxia in the environment (Felder, 1979; Thompson and Pritchard, 1969; Zebe, 1982; Anderson et al, 1994). The main objective of the present study is to evaluate a suite of physiological mechanisms that could underlie the anoxia tolerance of this species.

Generally speaking, decapod crustaceans use a variety of physiological and biochemical adjustments to compensate for reductions in ambient \( \text{PO}_2 \). In acute hypoxia, many increase the rate and depth of scaphognathite beating (to increase branchial water flow), increase cardiac output (mediated by either increasing heart rate or stroke volume), and shunt hemolymph flow away from the viscera (see Grieshaber et al., 1994; McMahon, 2001; Burnett and Stickle, 2001...
for review). In chronic hypoxia, many crustaceans abandon these energy-consuming systemic compensatory activities and begin modulating the carrying capacity of their respiratory pigments through synthesis of new hemocyanin subunits (Mangum, 1988; DeFur et al., 1990;) or allosteric regulation with various divalent cations, chloride, thiosulfate, neurohormones, and anaerobic end-products such as L-lactate and urate (Morris, 1990; Bridges, 2001; Menze et al., 2005b). As oxygen tensions decline to critical levels, these animals begin to reduce oxygen consumption, reduce energy expenditure, and recruit fermentative pathways for substrate-level ADP phosphorylation (Grieshaber et al., 1994).

Many ghost shrimps demonstrate one or more of these physiological and metabolic strategies, permitting them to thrive in the low-oxygen conditions of their habitats. Extremely low metabolic rates and critical $PO_2$ values (ambient $PO_2$ at which oxygen consumption decreases linearly with ambient oxygen, $P_{crit}$) have been observed in *L. louisianensis* (Felder, 1979) and *Neotrypaea californiensis* (formerly *Callianassa*) (Thompson and Pritchard, 1969; Torres et al., 1977), among others (see Stanzel and Finelli, 2004). Increased scaphognathite beat frequency in mild hypoxia followed by a reduction in that activity as severe depletion of oxygen occurs has been observed in *Calocaris macandreae* and *Trypaea australienensis* (Anderson et al., 1991; Paterson and Thorne, 1995). A similar pattern in heart rate has been observed in *N. californiensis* (Thompson and Pritchard, 1969). *N. californiensis* and *L. louisianensis* also increase their pleopod stroke rate as ambient $PO_2$ declines below normoxic values and return to resting rates when ambient $PO_2$ falls below their respective $P_{crit}$ values (Torres et al., 1977; Felder, 1979). Torres et al. (1977) observed that this behavior does not significantly contribute to oxygen uptake in a confined chamber in the lab; however, this behavior might be a natural response to draw water with higher ambient $PO_2$ into the burrow.
As ambient $PO_2$ approaches the respective $P_{crit}$ values of crustaceans, many species begin recruiting anaerobic fermentative pathways to provide ATP for metabolic processes. It is currently thought that anaerobic glycolysis terminating in the formation of lactate is probably the only source of ATP provision during severe hypoxia and anoxia in Crustacea (Albert and Ellington, 1985; Grieshaber et al., 1994). Generally, lactate fermentation is associated with low tolerance (short-term survival) under anoxia because the substrate, mostly glycogen, is rapidly consumed (Hochachka, 1980; Hagerman, 1998). This limitation may explain the inability of most decapods to withstand severe hypoxia or anoxia for more than a few hours. Unlike its decapod relatives, *L. louisianensis* and other thalassiniid shrimps survive anoxic conditions in the laboratory for several days (Thompson and Pritchard, 1969; Felder, 1979; Zebe, 1982). Though small amounts of alanine, aspartate, glutamate, succinate, and malate have been reported, L-lactate is the principle metabolic end-product measured in *N. californiensis* and *U. pugettensis* (Pritchard and Eddy, 1979; Zebe, 1982). Though unpublished observations suggest that lactate accumulates in copious amounts in *L. louisianensis*, no published data exist regarding the anaerobic metabolism of this species (Felder et al., 1995; Bourgeois and Felder, 2001). Thus, one goal of this research is to measure the amount of lactate produced by *L. louisianensis* under anoxic stress for up to several days.

Given that *L. louisianensis* survives nearly twice as long under anoxia and at warmer temperatures than *N. californiensis* and *U. pugettensis* (Felder, 1979), it is possible that anaerobic pathways in addition to lactate fermentation are recruited, resulting in the accumulation of several diverse metabolites. Several invertebrates, including freshwater snails, mussels, oysters, and lugworms have anaerobically functioning mitochondria that accumulate succinate and the volatile fatty acids propionate and acetate via the pathway known as malate
dismutase (see Van Hellemond et al., 1995; Tielens et al., 2002). In this pathway, malate is transported to the mitochondria where it is either oxidized to oxaloacetate (eventually yielding succinyl-CoA and acetate) or reduced to fumarate. The fumarate is then reduced to succinate, which is further metabolized to propionate (see Grieshaber et al., 1994 and Tielens et al., 2002 for details of this pathway). The reduction of fumarate is linked to the NADH dehydrogenase (Complex 1 of the electron transport chain), which pumps protons that facilitate ATP formation via the F\textsubscript{1}F\textsubscript{0}-ATP synthase. Unlike aerobic mitochondria, which transfer electrons through components of the ETC via ubiquinone, the transfer of electrons to fumarate under anoxia is accomplished with rhodoquinone, characterized by a much lower redox potential (Van Hellemond et al., 1995; Tielens et al., 2002). It has been postulated that this pathway is coupled with the metabolism of various branched-chain amino acids, leading to the accumulation of other VFAs including isovalerate, methylbutyrate, and isobutyrate (Lahoud et al., 1971; Hochachka, 1980; Holst and Zebe, 1986; Kita, 1992; Tielens, 1994). If rhodoquinone is detectable in physiologically relevant amounts in the mitochondria of L. louisiannensis, it is reasonable to speculate that volatile fatty acids could be synthesized in a similar manner in this animal.

The arginine phosphate/kinase system is another of the suite of mechanisms animals may use to tolerate anoxia and severe hypoxia in their environment (Ellington, 2001). Lutz et al., (1985) demonstrated in turtle brain slices subjected to anoxia that creatine phosphate buffered the depletion of ATP over a period of 120 min. Morris et al. (2005) demonstrated a similar role for arginine phosphate in the freshwater crayfish, Cherax detructor exposed to progressive hypoxia. These authors observed no change in the adenylate energy charge (AEC), ATP:ADP ratio, the total adenylate pool, or the adenylate kinase equilibrium in the tail and chelae muscle following the progressive hypoxia regime to which the crayfish were subjected. Only a 17%
decrease in the arginine phosphate pool was measured, suggesting other compensatory mechanisms contributed to the prevention of ATP depletion. When exposure to a heavy metal (lead) depressed the contribution of aerobic respiration to the maintenance of ATP supply, ADP increased significantly in both the chelae and tail muscle, causing a significant decrease in the AEC, lowering the ATP:ADP ratio, and lowering the value of the $K'_{ADEN}$ (Morris et al., 2005). Those changes occurred with no decrease in ATP, which the authors suggest was buffered by arginine phosphate, and by the third hour of hypoxia and lead exposure, all values returned to normoxic levels (Morris et al., 2005).

A second goal of this research is to test the hypothesis that arginine phosphate buffers the loss of ATP during the initial stages of anoxia in *L. louisianensis*. I predict that any decrease observed in the AEC and ATP/ADP ratio—at least in the initial hours of anoxia—will be manifested by increases in ADP concentration. Based on the current theory that the animals that are most successful at surviving anoxia do so by reducing metabolism to pilot-light levels (Hand, 1998; Hochachka and Lutz, 2001), I expect *L. louisianensis* to reduce energy expenditure to probably less than 10% of aerobic rates, resulting in a slow reduction of ATP over time. Though some authors have questioned the reliability of the AEC as a measure of energy metabolism in invertebrates (e.g., see Thebault et al., 2000; Morris et al., 2005), I will also measure the level to which AEC declines when this species is subjected to extended bouts of anoxia.

Another feature of mitochondria that could be linked to the duration of anoxia tolerance is the presence or absence of a mitochondrial permeability transition pore (MPTP). When oxygen is depleted in cells, many events occur that lead to a rise in intracellular calcium (Hochachka, 1986). In mammals, the MPTP opens in response to high calcium, and this event
promotes the release of cytochrome c, a key signaling component in the initiation of apoptosis and necrosis (Liu et al., 1996). Such a mitochondrial permeability transition does not occur in response to high calcium with mitochondria isolated from embryos of the anoxia-tolerant brine shrimp, *Artemia franciscana* (Menze et al., 2005c), even though the mitochondria possess all molecular components purported to constitute the MPTP in mammals. Menze et al. (2005c) report that these mitochondria resist MPTP opening when challenged with millimolar levels of calcium, and further, display a remarkably large capacity for calcium uptake and storage in the matrix. These authors postulate that the lack of a permeability transition may contribute to this animal's prolonged anoxia tolerance, which can extend to years (Clegg, 1997). The final aim of this thesis is to test for a mitochondrial permeability transition in *L. louisianensis*. If absent, such a result would support the proposal by Menze et al. (2005c) that the lack of a functional MPTP may be a common feature of crustacean, and more generally of invertebrates.
MATERIALS AND METHODS

Experimental Animals

Specimens of the ghost shrimp *Lepidophthalmus louisianensis* were collected during low tide from a mudflat near Waveland, MS (30°15'24.88"N, 89°24'54.46" W). The shrimp were flushed from their burrows by liquefying the sediment with a gas-powered water pump. This method yielded numerous shrimp, reduced the number of injured animals, and improved overall survivorship, when compared to the common method of extracting the shrimp from their burrows with negative pressure using a manual suction pump (‘Yabby’ pump). On occasion, the latter method was used when only small numbers of shrimp were needed.

To prevent aggressive interactions, the shrimp were kept separated in perforated plastic vials that were placed in large containers filled with seawater from the collection site (10-20 practical salinity units, PSU). Following a short quarantine and salinity-acclimation process, the animals contained in vials were transferred to an aquarium equipped with biological, chemical, and mechanical filtration, and plumbed with recirculating artificial seawater (ASW). ASW at a concentration of 20 PSU was used as prescribed by Felder (1978, 1979). The highest mortality (normally less than 5% of animals) occurred within 3 days of collection and was attributed to heavy parasitic infections or handling stress. The aquarium was isolated in a dark room with only brief periods of direct lighting.

Animals used in experiments were kept for a minimum of 3 days and no longer than 2 weeks and were not fed during this period. Intermolt males with no missing appendages and devoid of noticeable parasite infections were selected for experiments. Animals that exhibited black discoloration near the gills, contained roundworms (most commonly near the heart), or
large numbers of red spots on the carapace were not used. Occasionally, non-ovigerous females were combined with males to obtain sufficient quantities of tissue for mitochondrial studies.

Quantification of Anoxia Tolerance

In preliminary experiments, I observed that smaller shrimp (<1 g wet wt.) survived substantially longer in anoxic seawater than larger ones (>2 g wet wt.), an observation that prompted us to measure anoxia survival for two size groups. The length of the carapace (CL) from the tip of the rostrum to the posterior margin of the cardiac region was not measured for each animal, but I estimate that the smallest animals used had a CL > 10 mm. Thus all shrimp used were either sexually mature adults or within one molt of sexual maturity according to descriptions for this species (Felder and Lovett, 1989).

Large and small adult shrimp, contained in perforated, plastic vials, were placed inside biological oxygen demand (BOD) bottles (Felder, 1979). Animals were less active and expended less energy in locomotion when inside vials, perhaps because the vials simulated to a degree the confined microenvironment of burrows. Without vials, the shrimp swam continuously in circles and died much earlier under anoxia. The 300-ml BOD bottles were filled with anoxic artificial seawater (20 PSU), sealed with a glass stopper, and placed in a temperature-controlled (25°C) dark room. The seawater was made nominally anoxic by purging with N₂ for 1.5 h. Mortality was scored as the inability to stimulate animal movement (e.g., pleopod beating) by rotating the BOD bottle. In early studies, heart rate was also observed, but this second indicator did not enhance the reliability of mortality assessment. Once projected as dead, the shrimp was removed from its bottle and placed in oxygenated water to confirm the absence of recovery. Survivorship was checked every 5-8 h for the first 36 h of anoxia exposure, and 1-4 h thereafter. From these data, LT₅₀ values (lethal time for 50% mortality under anoxia)
were calculated. Control animals were kept in open BOD bottles or 100-ml culture tubes with periodic aeration of the seawater. In two separate experiments, the antifungal agent amphotericin B (2.5 mg/L) or an antibiotic cocktail of 5 mg/L chloramphenicol, 50 mg/L gentamycin, 100,000 units/L penicillin, and 100,000 µg/L streptomycin was added to the seawater to test whether anoxia tolerance of adult *L. louisianensis* could be extended by restricting growth of fungi or bacteria.

**Preparation of Animal Extracts**

Ghost shrimp were exposed to anoxia as above for 6, 12, 24, 48, and 72 h. At each time point, individuals were removed from their BOD bottles, blotted dry, freeze-clamped in liquid N$_2$, and ground into a fine powder with a pre-chilled mortar and pestle under liquid N$_2$. The frozen powder (~2g) was then homogenized in a ground-glass homogenizer containing 5 volumes of ice-cold 6% perchloric acid (PCA) with 10 mM sodium ethylenediaminetetraacetic acid (EDTA). The acid-insoluble fraction was removed by centrifugation for 20 min at 10,000 g and 4º C. The supernatant was then neutralized with ice-cold 5 M K$_2$CO$_3$ and centrifuged at 10,000 g for 10 min and 4º C to remove the potassium perchlorate precipitate. For arginine phosphate, identical procedures were used, but animals were limited to 48 h of anoxia, and one group was given a 24 h period of normoxic recovery. To measure adenylates, each PCA supernatant was divided into two equal aliquots. One aliquot was neutralized with ice-cold 5 M K$_2$CO$_3$ and the other with ice-cold 5 M K$_2$HPO$_4$, and then both were centrifuged as above to remove perchlorate salts. PCA extracts of tissues associated with molluscan and crustacean exoskeletons contain high amounts of Ca$^{++}$, which can cause significant precipitation of Ca$^{++}$-ATP and Ca$^{++}$-ADP upon neutralization of extracts with K$_2$CO$_3$ (Rees and Hand, 1991), even with EDTA added to the PCA. Unpublished observations suggest that neutralization with
K$_2$HPO$_4$ avoids this problem by preferentially precipitating the Ca$^{++}$ as CaPO$_4$ and leaving the adenylates in solution (C. Ortmann, pers. comm.; Heinrich-Heine University, Duesseldorf, Germany). Consequently, we compared the two neutralization procedures. All samples were stored at -80ºC until chemical analyses were performed. Upon thawing, any additional precipitate was removed by centrifugation.

**Enzyme Assays for Lactate and Arginine Phosphate**

L-lactate in PCA extracts was measured using a diagnostic kit (Trinity Biotech, Procedure No. 735, Wicklow, Ireland). With this method, the oxidation of lactate to pyruvate and H$_2$O$_2$ is catalyzed by lactate oxidase. Using the H$_2$O$_2$ produced, peroxidase catalyzes the oxidative condensation of chromogen precursors producing a colored dye with an absorption maximum at 540 nm in a concentration that is directly proportional to the concentration of lactate in the sample (Jackson *et al*., 2001). The absorbance change was recorded in 96-well plates using a Spectramax 384 plate reader (Molecular Devices, Sunnyvale, CA). Lactate samples measured with the traditional Bergmeyer protocol based on lactate dehydrogenase (Gutmann and Wahlefeld, 1974) gave identical results, but the Trinity Biotech procedure yielded much higher sample throughput.

Arginine phosphate was quantified using a two-step, enzyme-catalyzed reaction, and the absorbance recorded with a dual beam spectrophotometer (Cary 100 Bio, Varian, Walnut Creek, CA). The protocol was essentially the same as that described by Ellington (1989). The assay medium contained 50 mM imidazole/HCl (pH 7.0), 2 mM Mg acetate, 10 mM D-glucose, 0.5 mM ADP, 1 mM NADP, and 290 µl neutralized supernatant (diluted with deionized H$_2$O as needed). After baseline absorbance was recorded at 340 nm, 5 µl of hexokinase/glucose-6-phosphate dehydrogenase enzyme mixture (Roche Diagnostics, Mannheim, Germany) was added
and the absorbance change recorded. Once all endogenous ATP was consumed, 5 µl of arginine kinase (AK) was added, and the arginine phosphate concentration was determined from the change in absorbance as calibrated with ATP standards. AK was expressed from a pET 22b plasmid clone (Strong and Ellington, 1996), and the functional properties of this crustacean enzyme have been described previously (Pruett et al., 2003).

**HPLC Analyses of Adenylates**

Analyses were performed using a protocol modified from Menze et al., (2005a) with a Dionex HPLC system (Dionex, Sunnyvale, CA), which consisted of a PDA-100 photodiode array detector, GP-50 gradient pump, AS50 auto sampler, and AS50 thermal compartment. Samples were maintained at 4º C in the auto sampler, and 75 µl of each sample were applied to a 250 x 4.6 mm strong anion exchange column (Sphereclone 5µm SAX 80A, Phenomenex, Torrance, CA). The samples were eluted with a linear gradient from 40 mM K$_2$HPO$_4$ (pH 5.5) to 500 mM K$_2$HPO$_4$ (pH 5.5) over 26 min at a flow rate of 1 ml/min at 30ºC. The absorbance of the eluent was monitored with a photodiode array detector at wavelengths from 190 to 390 nm. Peaks were identified by analysis of the given peak spectrum from a recorded three-dimensional field with Chromeleon software (Dionex) and by comparison with retention times of standards. Concentrations of AMP, ADP, and ATP were determined from peak area at 260 nm using Chromeleon software. Calibration curves were linear from 1 µM to 1 mM.

**Isolation of Mitochondria**

Mitochondria were isolated from hepatopancreas tissue of shrimp using a protocol similar to Menze et al., 2005c. Hepatopancreas tissue was dissected from 25-30 shrimp (ca. 5 g of tissue) and pooled in 40 ml of ice-cold isolation buffer 1 [0.3 M sucrose, 150 mM KCl, 1 mM EGTA, 0.5% (wt/vol) fatty acid-free BSA, and 20 mM K$^+$-HEPES, pH 7.5]. The isosmotic
pressure (750 mOsm) of isolation media for *L. louisianensis* mitochondria is based on the osmotic and ionic properties of the hemolymph of these shrimp (Felder, 1978). The tissue was homogenized in a glass-Teflon homogenizer (Thomas Scientific, Swedesboro, NJ) at 1,000-1,100 rpm for six passages. The homogenate was centrifuged for 10 min at 1,000 g and 4º C to pellet the cellular debris. The supernatant was removed and centrifuged at 9,000 g for 15 min to pellet the mitochondria fraction. The resulting pellet was then resuspended in ice-cold isolation buffer 2 [0.3 M sucrose, 150 mM KCl, 0.025 mM EGTA, 0.5% (wt/vol) fatty acid-free BSA, and 20 mM K⁺-HEPES, pH 7.5] and centrifuged again at 9,000 g and 4º C. This wash and centrifugation step was repeated, and the final pellet was resuspended in ~1 ml of isolation buffer 2, which gave a protein concentration of about 8-12 mg protein/ml. Protein was quantified using Coomassie Plus™ protein assay kit (Pierce, Rockford, IL) with BSA as the standard.

**Mitochondrial Volume Change**

Changes in volume of isolated mitochondria were measured spectrophotometrically as described in Menze *et al.* (2005c) and previously developed by (Petronilli *et al.*, 1993). A decrease in absorbance at 540 nm is indicative of an increase in mitochondrial volume (swelling). Measurements of mitochondria (0.5 mg protein/ml) were carried out at 25 ºC, and the reaction medium contained 300 mM sucrose, 150 mM KCl, 1 mM KH₂PO₄, 5 µM rotenone, 25 µM EGTA and 20 mM K-HEPES, pH 7.5. Swelling was induced by addition of HgCl₂. Calcium does not induce a detectable change in permeability of these mitochondria (see Results). Studies with energized mitochondria were performed in the presence of 5 mM succinate.

**Fluorescence-Based Measurements of Calcium**

Calcium-induced fluorescence was measured according to Menze *et al.* (2005c). Assays for calcium uptake by mitochondria were carried out in 96-well plates in a fluorescence plate
reader (Victor 3, PerkinElmer Inc., Wellesley, MA) at 25 °C in the reaction medium described for the swelling assay above. Studies with energized mitochondria were performed in the presence of 5 mM succinate. Mitochondria were de-energized in the absence of succinate by the addition of the uncoupler FCCP (Carbonyl cyanide p-trifluoromethoxyphenylhydrazone), which serves to abolish the membrane potential. As a control, calcium fluorescence was also measured for isolation buffer 2 without mitochondria. The calcium-sensitive fluorescence probe, fluo-5N, was added at a final concentration of 1 mM (Blattner et al., 2001), and fluorescence was excited from above the wells 20 min after addition of calcium at concentrations of 0.1, 0.2, 0.3, 0.6, and 1.0 mM. The green fluorescence of fluo-5N was measured using an excitation filter of 485 nm and an emission filter of 535 nm. Fluorescence was expressed as percent of the maximal fluorescence (% $F_{\text{max}}$) obtained when the calcium probe was completely saturated with calcium in the respective buffer system. To obtain $F_{\text{max}}$ the fluorescence was measured at intervals across a range of 1 to 1000 µM calcium. Data were fitted to the function: $F = (K \cdot X \cdot F_{\text{max}}) / (1 + K \cdot X)$ using the computer software SigmaPlot Version 9.01 (SPSS Inc., Chicago, IL). In this function, F is the measured fluorescence, K is the stoichiometric binding constant (1/K$_d$) of calcium to fluo-5N, X is the calcium concentration, and $F_{\text{max}}$ is the maximal obtainable fluorescence. The dissociation constant K$_d$ estimated was about 100 mM and within the range stated by the supplier (Menze et al., 2005c).

Measurement of Rhodoquinone:Ubiquinone Ratio

Isolated mitochondria were prepared as described above, except that the final wash and 9000 g centrifugation were omitted and only isolation buffer 1 was used. The final mitochondria pellet was resuspended in 0.5 mL of isolation buffer 1. The sample was frozen in liquid N$_2$, lyophilized, and sealed under N$_2$ gas. The sample was shipped to Professor Louis Tielens.
(Utrecht University, Utrecht, The Netherlands) for analysis of rhodoquinone and ubiquinone by HPLC and mass spectrometry.

Statistical Analyses

Graphpad Prism 4.0 software was used to create survival curves for LT$_{50}$ measurements. The non-parametric Kaplan-Meier test was used to estimate Log-Rank and Wilcoxon values for comparing the survival curves (Kaplan and Meier, 1958). A confidence level of 95% was used to test for significant differences between curves. LT$_{50}$ values (median survival times) were estimated using the trimmed Spearman-Karber method (alpha was 10%) (Hamilton et al., 1977).

SigmaStat 3.0 software was used to perform all other statistical analyses. For lactate, arginine phosphate, adenylate, and calcium uptake data, all tests for normality (Kolmogorov-Smirnov test) failed. Therefore, a Kruskal-Wallis ANOVA on ranks test was used. When differences within a treatment group were detected, the Tukey method was used for multiple comparisons between individual time points.
RESULTS

Anoxia Tolerance

Survival under anoxia was evaluated independently for two size classes of *L. louisianensis*. Both size classes exhibited remarkable capacities for anoxia tolerance, with all animals surviving at least 24 h. The LT$_{50}$ for large shrimp (> 2 g) was 64.8 h (Figure 1) with one shrimp surviving 92 h without oxygen. The addition of the fungicide amphotericin B or an antibiotic cocktail did not improve the LT$_{50}$ for large shrimp (data not shown). In contrast, the LT$_{50}$ for small shrimp (<1g) was a striking 113 h, which was nearly twice that of the large shrimp (Figure 1). Eleven of 17 animals survived for 100 h, and two animals in this group lived more than 160 h (almost one week) in the absence of oxygen. Control animals for both size classes suffered little mortality during these trials, with less than 15% dying within 10 days and most living beyond 3 to 4 weeks (data not shown).

Metabolite Levels during Anoxia

Lactate accumulation in *L. louisianensis* (large shrimp only) is plotted in Figure 2. Lactate in whole-animal extracts was negligible in the control group (0.42 ± 0.39 µmoles g.f.w.$^{-1}$, mean ± SD, N=6) but rose 18-fold in animals exposed to anoxia for 6 h (18.4 ± 5.38 µmoles g.f.w.$^{-1}$). After 12 h of anoxia, lactate reached levels more than 40 times that of control animals and more than 60 times that of the controls after 24 h. Between 24 and 48 h, lactate levels nearly doubled from 65.2 ± 5.38 to 118 ± 17.0 µmoles g.f.w.$^{-1}$. Lactate concentration appeared to reach a plateau after 48 h with no significant increase observed at 72 h of anoxia. Lactate concentration reached similar levels in small shrimp, but data are not shown because a comprehensive analysis of that size group was not performed.
Figure 1. Survival curves for *L. louisianensis* under anoxia for two size classes. The LT$_{50}$ for large specimens (>2 g, N=22) was 64 h and for small animals (<1 g, N=17) was 113 h. LT$_{50}$ values were estimated using the trimmed Spearman-Karber method. The non-parametric Kaplan-Meier test was used to estimate Log-Rank and Wilcoxon values for comparing the survival curves (P<0.0001).
Figure 2. Lactate accumulation in *L. louisianensis* during a 72-h time course under anoxia. Lactate increases significantly at each time point until 72 h. Matching letters indicate groups that are not significantly different. Each bar represents mean ± SD for n=6 experiments. (One-Way ANOVA, Tukey pair-wise comparison, P<0.001).
The pool of arginine phosphate in *L. louisianensis* declined slowly across the anoxia time course and likely contributed to the stability of the adenylate energy charge (AEC) during this period (Figure 3). Arginine phosphate was significantly lower than the control value after 12, 24 and 48 h of anoxia. Arginine phosphate declined to a low of $3.78 \pm 0.93 \mu$moles g.f.w.$^{-1}$ by 48 h, which represents more than a 50% reduction from control levels ($8.22 \pm 0.45 \mu$moles g.f.w.$^{-1}$). The pool of arginine phosphate returned to control levels by 24 h post-anoxia.

It is noteworthy that statistically-significant differences in AMP, ADP, or ATP were not detected over the duration of the anoxia exposures (Table 1). Neither were there statistical changes in the total pool of adenylates, ATP:ADP ratio, AMP:ATP ratio, or the AEC (Figure 3). The metabolically-active tissues of *L. louisianensis* possess the capacity to fully defend the energetic status of the adenylate pool during the 48 h bout of anoxia.

The method by which perchloric acid extracts were neutralized had a profound effect on adenylate levels measured in the samples. An example is shown in Figure 4, where very little ATP was detectable in an extract neutralized with 5 M $\text{K}_2\text{CO}_3$, yet the same extract (equal volume derived from the same animal) neutralized with $\text{K}_2\text{HPO}_4$ contained substantially more ATP. This large difference was not observed for all extracts, but was sufficiently frequent that the adenylate data set collected with $\text{K}_2\text{CO}_3$ neutralization was discarded. The most likely reason for the large adenylate variability seen with $\text{K}_2\text{CO}_3$ is high and variable contents of calcium among samples, which caused precipitation of ATP and ADP during neutralization unless prevented by the presence of phosphate ion. Calcium binds tightly to phosphate and forms calcium phosphate precipitates.

Potential end-products of anaerobic metabolism other than lactate were not investigated primarily due to the finding that *L. louisianensis* does not contain physiologically-significant
Figure 3. Arginine phosphate concentration (bars) and adenylate energy charge (line) in adult *L. louisianensis* during a 48-h time course under anoxia and a 24-h recovery period under normoxia. Matching letters indicate groups that are not significantly different. Each bar represents mean ± SD (N=4 for recovery, N=5 for all other groups). One-way ANOVA with Holm-Sidak pair-wise comparison (P<0.001) was used for statistical comparison.
Table 1. Adenylate levels in *L. louisianensis* exposed to a time course of anoxia. Post-anoxia (recovery) animals were provided with aeration after 48 h of anoxia. Values are reported in micromoles per gram fresh weight. Means ± one standard deviation (N=3) are given for all treatment groups. No significant differences were observed across experimental treatments for any adenylate value; the P values for comparisons of means were all greater than 1.48 (One-Way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>6 hour</th>
<th>12 hour</th>
<th>24 hour</th>
<th>48 hour</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>0.63 ± 0.16</td>
<td>1.55 ± 1.74</td>
<td>0.59 ± 0.15</td>
<td>0.69 ± 0.19</td>
<td>1.40 ± 0.82</td>
<td>0.86 ± 0.45</td>
</tr>
<tr>
<td>ADP</td>
<td>7.85 ± 1.51</td>
<td>9.23 ± 2.03</td>
<td>7.66 ± 0.42</td>
<td>8.10 ± 0.77</td>
<td>9.05 ± 1.34</td>
<td>6.49 ± 0.32</td>
</tr>
<tr>
<td>ATP</td>
<td>73.6 ± 5.26</td>
<td>61.1 ± 6.33</td>
<td>62.8 ± 10.4</td>
<td>59.6 ± 13.2</td>
<td>56.3 ± 2.57</td>
<td>60.7 ± 4.95</td>
</tr>
<tr>
<td>SUM</td>
<td>82.1 ± 5.19</td>
<td>78.3 ± 10.5</td>
<td>71.5 ± 11.0</td>
<td>68.4 ± 12.6</td>
<td>56.8 ± 2.56</td>
<td>68.01 ± 5.31</td>
</tr>
<tr>
<td>ATP:ADP</td>
<td>9.6 ± 1.83</td>
<td>7.15 ± 1.44</td>
<td>8.23 ± 0.95</td>
<td>7.46 ± 2.16</td>
<td>6.32 ± 1.02</td>
<td>9.35 ± 0.39</td>
</tr>
<tr>
<td>AMP:ATP</td>
<td>0.01 ± 0.00</td>
<td>0.02 ± 0.02</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.01</td>
<td>0.03 ± 0.02</td>
<td>0.01 ± 0.01</td>
</tr>
</tbody>
</table>
Figure 4. Analysis of adenylate levels in *L. louisianensis*. Representative chromatograms from neutralization of a perchloric acid extract from the same animal with KH$_2$PO$_4$ (A) and K$_2$CO$_3$ (B). Neutralization with KH$_2$PO$_4$ fosters substantially higher recovery of ATP. See text for further explanation.
levels of rhodoquinone (A. Tielens, pers. comm.; data under analysis). Though a small amount of rhodoquinone was detected, the ratio to ubiquinone was exceedingly low. The presence of rhodoquinone is a prerequisite for mitochondrial-based pathways utilized in some species under anoxia to expand the ATP yield and increase the diversity of end products produced (see Introduction).

Mitochondrial Swelling and Calcium Uptake

Previous studies have shown that addition of calcium initiates a mitochondrial permeability transition in mammals (Hunter et al., 1976; Hunter and Haworth, 1979; Menze et al., 2005c). It is well-documented that the opening of the mitochondrial permeability transition pore leads to the release of calcium stored in the matrix of the mitochondria, as well as the release of important signaling molecules for the initiation of apoptosis and necrosis (Green and Reed, 1998; Zamzami and Kroemer, 2001; Green and Kroemer, 2004; Saelens et al., 2004; Bernardi et al., 2006). This permeability transition is manifested in a swelling that can be observed as a decrease in the absorbance of mitochondria at 540 nm (Petronilli et al., 1993). Absorbance actually increases when 1 mM calcium is added exogenously to energized mitochondria isolated from hepatopancreas tissue in L. louisianensis (Figure 5). The absence of a decrease in absorbance suggests that mitochondria from L. louisianensis do not experience an MPTP opening under physiological conditions that would trigger MPTP opening in mammals (cf. Menze et al., 2005c). The increase in absorbance observed is indicative of the transport of large quantities of calcium into the matrix (see Discussion). Addition of 20 µM HgCl₂, a non-physiological inducer of mitochondrial swelling, causes a profound decrease in absorbance of these mitochondria as expected (Figure 5). The mercury tracing serves as a positive control,
which indicates that if swelling had been induced by calcium-dependent opening of the MPTP, the assay was apparently adequate for detection.

A high capacity for calcium uptake was observed for energized mitochondria (~0.5 mg protein/mL) isolated from this anoxia-tolerant invertebrate. Because the fluo-5N dye cannot penetrate the mitochondrion, the fluorescence values measured and expressed as %F$_{\text{max}}$ correlate with the external free calcium concentration for values below the saturation signal of the calcium probe. In the absence of exogenously-added calcium, fluorescence was detected in solutions containing de-energized and energized mitochondria, as well as in the control without mitochondria, which indicates some calcium contamination is present on the glassware and in the mitochondrial preparations. At each concentration of exogenously added calcium, the fluorescence for de-energized preparations of mitochondria is statistically the same as the control. These results indicate that calcium is not taken up or released by the de-energized mitochondria. However, energized mitochondria significantly reduce the level of exogenously added calcium below the control at each concentration investigated. Above 0.2 mM exogenous calcium, the free calcium levels begin to rise in energized mitochondrial preparations, but even at 1 mM calcium, fluorescence remains significantly lower than that of the control (Figure 6). Thus, the capacity for calcium uptake by ghost shrimp mitochondria is very high. Even more noteworthy, the lack of calcium-induced release of calcium from the mitochondrial matrix, for either energized or de-energized mitochondria, supports the absence of a functional MPTP in L. louisianensis mitochondria.
Figure 5. Effects of Ca\textsuperscript{2+} and HgCl\textsubscript{2} in the presence of 1 mM phosphate on volume changes in isolated mitochondria from hepatopancreas tissues of *L. louisianensis*. Mitochondrial swelling (i.e., a decrease in absorbance) is not observed after addition of 1 mM CaCl\textsubscript{2} (trace a) to energized mitochondria (0.8 mg protein/mL in 300 mM sucrose, 150 mM KCl, 1 mM KH\textsubscript{2}PO\textsubscript{4}, 5 \textmu M rotenone, 25 \textmu M EGTA, and 20 mM K-HEPES, pH 7.5). Mitochondrial volume increases after addition of HgCl\textsubscript{2} (trace b).
Figure 6. Ca\textsuperscript{2+} uptake by isolated mitochondria from hepatopancreas of *L. louisianensis*. Mitochondria (~0.8 mg protein/mL) were incubated in exogenously added Ca\textsuperscript{2+} in the presence of the Ca\textsuperscript{2+} probe fluo-5N. Measured fluorescence was expressed as a percentage of the total fluorescence when the dye is saturated with Ca\textsuperscript{2+} (%F\textsubscript{max}). Energized mitochondria reduced the concentration of exogenously added Ca\textsuperscript{2+} at all concentrations investigated. Each bar represents mean ± SD of N=4 experiments. *Significantly different from control (ANOVA, Tukey pairwise comparison, P < 0.001)*
DISCUSSION

I have shown in this study that *L. louisianensis* has a remarkable tolerance for anoxia, and that they accumulate extremely high concentrations of lactate when exposed to chronic anoxia. To my knowledge, the lactate concentrations observed after 72 h of anoxia (over 125 micromoles/g fresh weight) are the highest ever reported for a crustacean species (e.g. Zebe, 1982; Albert and Ellington, 1985; Taylor and Spicer, 1987; Hill *et al*., 1991; Atkinson *et al*., 1994; Henry *et al*., 1994; Adamczewska and Morris, 2001). My data also suggest that this species may utilize, to a minor degree, reserves of arginine phosphate to buffer losses in ATP and maintain a constant adenylate energy charge. However, a far more substantial contribution to the maintenance of energetic status comes from the high rate of anaerobic glycolysis. Using simple calculations based on changes in lactate and arginine phosphate in this study and measurements of the resting metabolic rate (*MO₂*) of similar-sized animals by Felder (1979), I show that *L. louisianensis* maintains its ATP production rate at near-aerobic levels during the initial 12 h of anoxia, and between 12 and 48 h of anoxia, still supports about 50% of its aerobic metabolism. Only after 48 h, does a major depression of metabolism occur, down to less than 5% (Table 2). Based on these data, it appears that down regulation of metabolism is delayed in *L. louisianensis* until high levels of lactate accumulation are reached. This metabolic approach may allow the shrimp to continue substantial metabolic activity through 48 h. Behaviorally, this delay in downregulation may allow the animal to continue irrigating its burrow to draw in water with higher ambient *PO₂* for several hours into the anoxia episode. Finally, calcium plus phosphate does not trigger opening of the regulated MPTP in mitochondria from *L. louisianensis* as it does in mammalian mitochondria (e.g., see Menze *et al*., 2005c)
Table 2. Percentages of resting aerobic metabolism maintained under anoxia in *L. louisianensis*. Units for lactate, ATP and arginine phosphate are micromoles/g fresh weight calculated for the indicated time intervals. ATP yield from aerobic respiration is calculated from the data of Felder (1979), which reports respiration rates between 50 and 68 μl O₂/(g.f.w. * h) for animals ranging from 1.45 to 7.13 g. Rates were converted to μmol O₂/(g.f.w. * h), and then a P:O ratio of 2 was used to calculate ATP synthesis. Animals in the present study were incubated at 25°C, and sizes ranged from 2 to 5 g.

<table>
<thead>
<tr>
<th>Time in anoxia (h)</th>
<th>Lactate produced</th>
<th>ATP yield from lactate</th>
<th>Arg-P used</th>
<th>ATP yield from Arg-P</th>
<th>Total ATP yield</th>
<th>ATP yield via aerobic respiration</th>
<th>% contribution of anaerobic metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 – 6</td>
<td>18.4</td>
<td>36.8</td>
<td>- 1.8</td>
<td>1.8</td>
<td>38.6</td>
<td>72.9 - 53.5</td>
<td>53 - 72</td>
</tr>
<tr>
<td>6 - 12</td>
<td>26.2</td>
<td>52.5</td>
<td>- 2.0</td>
<td>2.0</td>
<td>54.5</td>
<td>72.9 - 53.5</td>
<td>75 - 100</td>
</tr>
<tr>
<td>12 - 24</td>
<td>20.6</td>
<td>41.1</td>
<td>- 0.5</td>
<td>0.5</td>
<td>41.6</td>
<td>145.8 - 107</td>
<td>29 - 39</td>
</tr>
<tr>
<td>24 - 48</td>
<td>53.3</td>
<td>107</td>
<td>- 0.1</td>
<td>0.1</td>
<td>107</td>
<td>292 - 214</td>
<td>36 - 49</td>
</tr>
<tr>
<td>48 - 72</td>
<td>3.8</td>
<td>7.6</td>
<td>-----</td>
<td>-----</td>
<td>7.6</td>
<td>292 - 214</td>
<td>3 - 4</td>
</tr>
</tbody>
</table>
Survival under Anoxia

The mean survival time ($LT_{50}$) of *Lepidophthalmus louisianensis* under anoxia in our study was 64.8 h, which is slightly lower than the 3.2 days (~76 h) reported for similar-sized animals of the same species under identical conditions by Felder (1979). This small difference is likely population-specific. The collection site in the Felder study was a tidally-influenced pond on Grand Terre Island, LA that only receives water from the ocean via a drainage ditch during high tides. Interestingly, both of these independent studies reveal a higher $LT_{50}$ for this species than what is reported for other thalassinideans under anoxia. For example, *Calocaris macandreae* specimens survive only 43 h at 10º C (Anderson et al, 1994). Specimens of *Neotrypaea californiensis* were originally reported to survive 138 h without oxygen at 10º C (Thompson and Pritchard, 1969), but later were found to survive only 52-60 h at 12º C (Zebe, 1982). The warmer habitats of *L. louisianensis* may experience more frequent and longer episodes of anoxia than the colder-water habitats. Whatever the case, no other decapods have been shown to survive such extended periods of anoxia in the laboratory.

Though no behavioral activities were quantified in this study, it is appropriate to note, at least qualitatively, the response of these animals when placed in anoxia. Once the bottle was sealed, most animals retreated to the bottom of the tube in which they were contained. Some shrimp remained active for several minutes after being sealed in the BOD bottle, but eventually settled into a quiescent state. All animals reduced locomotion and appendage movements to minimal levels and only moved when disturbed. This behavioral quiescence is typical of many species during oxygen limitation and presumably contributes to reduced energy expenditures (e.g., see Hand and Hardewig, 1996).
The smaller size class of ghost shrimp (<1 g) used in this study survived anoxia nearly twice as long as the larger shrimp. The basis for this result is unclear at present. One possibility is that the smaller animals possess a greater capacity for metabolic depression than larger ones, and thus glycogen stores would presumably last longer during subsistence-level metabolism.

Our biochemical data were restricted to the larger size class for the practical reason of ample quantities of tissue, but it would be interesting in future studies to follow metabolite changes in small animals, and to also compare rates of metabolic heat dissipation between large and small specimens under anoxia. An alternative explanation is that the result is an experimental artifact, arising from the fact that both size classes were exposed to anoxia in the same volume (300 ml) of water. One could argue that higher concentrations of waste products (e.g., protons, ammonia) might accumulate in containers with the larger animals and reduce their survivorship. When Felder (1979) replaced anoxic water daily, the LT50 for *L. louisianensis* increased from 3.2 to 4 days.

Treatment of the clam, *Macoma balthica*, with the antibiotic chloramphenicol significantly increased the LT50 in anoxia from 4.8 to 13.3 days (de Zwaan *et al.*, 2001). The same treatment extended the LT50 in anoxia of the clam, *Chamelea gallina*, from 2.1 to 11.0 days (de Zwaan *et al.*, 2002). Use of chloramphenicol, gentamycin, and penicillin/streptomycin did not significantly change the LT50 for *L. louisianensis* in anoxia, nor did the addition of amphotericin B.

**Anaerobic Metabolism**

Whole-animal analysis of lactate during selected periods of anoxia exposure revealed extremely high concentrations of this end product of anaerobic metabolism (Figure 2). Lactate values have not been reported previously for this species under anoxia, although commentary has
previously indicated that lactate production occurs in this species (Felder et al., 1995; Borgeois and Felder, 2001). Lactate accumulation has been quantified in a few other thalassinidean shrimp, but many of the early studies on this examined only hemolymph lactate. Lactate accumulated slowly in tissues of *C. macandreae* and *N. californiensis*; values reached $16.8 \pm 0.28 \mu$moles g.f.w.$^{-1}$ by 24 h in *N. californiensis* (Zebe, 1982) and $11.6 \mu$moles g.f.w.$^{-1}$ by 18 h in *C. macandreae*. Comparatively, *L. louisianensis* produced nearly 3-fold more lactate ($65.2 \pm 9.64 \mu$moles g.f.w.$^{-1}$) by 24 h of anoxia, albeit at a much higher ambient temperature (Figure 2). By 48 h, the concentration in *L. louisianensis* nearly doubled ($118 \pm 17.0 \mu$moles g.f.w.$^{-1}$). Temperature alone could account for a considerable fraction of the difference in lactate accumulation among these species of thalassinidean shrimp. One would predict that survival under anoxia would increase and the rate of lactate accumulation would decrease in *L. louisianensis* at lower temperatures. It would be interesting in future studies to investigate the Q$_{10}$ effect on the accumulation of lactate under anoxia in *L. louisianensis*.

Excretion of lactate into the water has been observed in selected crustacean species (de Zwaan and Skjoldal, 1979), but very little lactate is excreted into the water by *N. californiensis* or *C. macandreae* (Zebe, 1982; Anderson et al., 1994). The crayfish, *Austropotamobius pallipes*, accumulates lactate in its exoskeleton during lactic acidosis, which might aid in buffering of the extracellular fluid during anaerobiosis (Jackson et al., 2001). It would be interesting to determine if *L. louisianensis* has similar capabilities.

**Depletion of Arginine Phosphate and Maintenance of Adenylate Pools**

Phosphagen systems such as creatine phosphate and arginine phosphate have been shown to buffer the loss of ATP during functional and environmental anaerobiosis (reviewed in Grieshaber et al., 1994 and Ellington, 2001). This mechanism may be operative to some degree
in the maintenance of adenylate levels and the adenylate energy charge (AEC) in *L. louisianensis* (Table 1 and Figure 3). A 21% reduction in arginine phosphate was observed in the first 6 h of anoxia, while no significant change was observed in any of the adenylates. By 48 h, the arginine pool was reduced by 54% in the face of a stable ATP values. However, considering the small absolute drop in arginine phosphate, the quantitative contribution to adenylate stability is minor, when compared to the large ATP yield from lactate (Table 2).

A full analysis of anaerobic substrates and calorimetric measurements of heat dissipation during anoxia are needed to determine whether lactate production and arginine phosphate use are sufficient to fully satisfy ATP demand under anoxia in *L. louisianensis*. However, the calculations in Table 2 indicate that these two sources of ATP under anoxia can account for a sizable fraction of the resting aerobic metabolic rate. In other words, at least during the early stages of anoxia, metabolic rate is not greatly depressed compared to normoxia. This observation is surprising to me. From 0 – 6 h, it appears that the shrimp can sustain up to 72% of the aerobic rate of ATP generation from lactate and arginine phosphate metabolism. Between 6 and 12 h anoxia, the values increased to 100%. Finally, between 12 and 48 h, there appears to be a substantial metabolic depression under anoxia compared to normoxia (metabolism reduced to 30 – 50 % of resting aerobic rate). Deep metabolic depression under anoxia occurs between 48 and 72 h of anoxia, when the calculated metabolism is only 3-4% of the resting normoxic rate. Metabolic depression to 5-10% under anoxia in anoxia-tolerant invertebrates is commonly observed, and the degree of depression is strongly correlated with anoxic survivorship (Hand, 1998). An animal capable of surviving more than a few hours of anoxia would be expected to reduce metabolism to pilot-light levels for the duration of anoxia exposure (Hochacka and Lutz, 2001). On the other hand, it may not be so surprising that metabolic rate under anoxia is not
immediately reduced in such a tolerant species given the consideration that the resting metabolic rate in normoxia for *L. louisianensis* is well below values reported for several other crustaceans (reviewed in: Mangum, 1977; McMahon and Wilkens, 1983; McMahon, 2001).

**Absence of a Regulated MPTP**

My data demonstrate that 1 mM calcium does not induce swelling in the mitochondria from *L. louisianensis* and suggests that a regulated MPTP does not exist. Instead, addition of calcium caused an increase in absorbance, i.e., an apparent decrease in mitochondrial volume (Figure 5). Actually, it is unlikely that any shrinkage occurred, but rather that the formation of Ca$^{2+}$-phosphate complexes in the matrix during high calcium uptake caused an increase in the refractive index of the matrix (Andreyev et al., 1998; Chalmers and Nicholls, 2003; Nicholls and Chalmers, 2004). Addition of high concentrations of mercury (20 micromolar) to mitochondria has been shown to induce mitochondrial permeabilization by unspecific “damage” of membrane proteins that leads to opening of an un-regulated pore (He and Lemaster, 2002). Mercury caused substantial swelling of mitochondria of *L. louisianensis* (Figure 5). This observation serves as an important positive control by documenting the capacity of ghost shrimp mitochondria to swell and our ability to detect it. The absence of a MPTP is confirmed by the calcium uptake/release data in Figure 6, which shows that across a wide range of added calcium, release of matrix calcium (diagnostic of MPTP opening) does not occur in *L. louisianensis* mitochondria. In fact mitochondria of *L. louisianensis* continue to actively load calcium even when challenged with 1 mM extra-mitochondrial calcium.

The lack of a calcium-induced permeability transition and the high calcium uptake capacity was also observed in *Artemia franciscana* (Menze et al., 2005c) and may be a general feature of invertebrates. MPTP opening is a key step in the initiation of apoptosis and cell death.
in the hypoxia-sensitive mammals and is triggered by high levels of intracellular calcium, in addition to other signals (Ichas and Mazat, 1998; Jiang and Wang, 2004). Briefly, opening of the MPTP in mammalian mitochondria causes swelling, which then ruptures the outer membrane and releases cytochrome c. In mammals, cytochrome c then stimulates caspase-dependent apoptosis by binding to Apaf-1. The complex then can recruit and activate caspases (Menze et al. 2005c, and articles cited therein). Elevated calcium is a hallmark of many cell types exposed to prolonged anoxia (Hochachka, 1986). It could be interpreted that the lack of MPTP opening under conditions of high calcium may be a predisposition for extended anoxia tolerance in non-mammalian species.

In summary, the anoxia tolerance of the ghost shrimp *L. louisianensis* is among the highest, if not the highest, ever reported for a decapod species. Surprisingly, the animal exhibits only modest metabolic depression during the first 12 h. The depression is significantly delayed compared to other invertebrates, but eventually low metabolic rates (3-4% of normoxic values) are reached after 48 h. Lactate production is very high in tissues and appears to be the primary source of anaerobic ATP generation as in other crustaceans, which is supported in *L. louisianensis* by the very low levels of rhodoquinone. The lack of a regulated MPTP in *L. louisianensis* may be a general feature of invertebrates that contributes to an extended anoxia tolerance.
LITERATURE CITED


VITA

Jeremy D. Holman was raised in the rural town of Tipton, Oklahoma, and graduated from high school in Clinton, Tennessee, in 1994. Shortly after graduation, Jeremy joined the United States Navy and was trained as a radioman and data processor. Jeremy sailed on two deployments in the Western Pacific and Indian Oceans onboard the U.S.S. Cleveland, LPD-7 where he earned the prestigious Navy Achievement Medal for his service as the Leading Petty Officer in a division of 28 sailors, and as the primary technician for the construction of all radio and satellite telecommunications circuits onboard the ship.

Jeremy was honorably discharged from the U.S. Navy in 1999. In August of 2002, he began his undergraduate training at the University of North Texas, where he majored in biological sciences and did research under the tutelage of Dr. Warren Burggren and Dr. Pamela Padilla. During the summers of 2002 and 2003, Jeremy worked as an NSF-REU Fellow and research associate at the Grice Marine Laboratory in Charleston, South Carolina, under the supervision of Dr. Lou Burnett, where he published his first and second journal articles. Jeremy graduated from the University of North Texas with honors on May 11, 2004.

Jeremy began his graduate training at Louisiana State University in August of 2004, in the lab of Dr. Steven Hand. In his thesis, Jeremy examines the anoxia tolerance and anaerobic metabolism of the ghost shrimp, *Lepidophthalmus louisianensis*. He learned that this burrowing crustacean accumulates the highest levels of lactate ever recorded in a decapod species, and maintains near-aerobic levels of ATP during 48 hours without oxygen. Further, this shrimp lacks a calcium-sensitive mitochondrial permeability transition pore, which may aid in protecting the mitochondria from programmed cell death during times of energy limitation such as in anoxia.