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Metabolic Depression is Delayed and Mitochondrial Impairment Averted during Prolonged Anoxia in the ghost shrimp, *Lepidophthalmus louisianensis* (Schmitt, 1935)

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Abstract

Lepidophthalmus louisianensis burrows deeply into oxygen-limited estuarine sediments and is subjected to extended anoxia at low tides. Large specimens (>2 g) have a lethal time for 50% mortality (LT₅₀) of 64 h under anoxia at 25° C. Small specimens (<1 g) have a significantly higher LT₅₀ of 113 h, which is the longest ever reported for a crustacean. Whole body lactate levels rise dramatically under anoxia and exceed 120 μmol g.f.w.⁻¹ by 72 h. ATP, ADP, and AMP do not change during 48 h of anoxia, but arginine phosphate declines by over 50%. Thus arginine phosphate may help stabilize the ATP pool. Surprisingly, when compared to the aerobic resting rate, ATP production under anoxia is unchanged during the first 12 h, and drops to only about 50% between 12 and 48 h. Finally, after 48 h of anoxia, a major metabolic depression to less than 5% occurs. Downregulation of metabolism is delayed in *L. louisianensis* compared to many invertebrates that exhibit facultative anaerobiosis. Bioenergetic constraints as a result of eventual metabolic depression led to ionic disturbances like calcium overload and compromised membrane potential of mitochondria. Because these phenomena trigger apoptosis in mammalian species, we evaluated the susceptibility of ghost shrimp mitochondria to opening of the mitochondrial permeability transition pore (MPTP) and associated damage. Energized mitochondria isolated from hepatopancreas possess a pronounced capacity for calcium uptake. Exogenous calcium does not stimulate opening of the MPTP, which potentially could reduce cell death during prolonged anoxia.

Keywords

Metabolic depression; anoxia; lactate; adenylates; cell death; mitochondrial permeability transition pore

1. Introduction

The ghost shrimp, *Lepidophthalmus louisianensis* (formerly *Callinassa 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.*

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louisianensis are found in low-energy beaches, back-beach ponds, estuarine tidal flats, and tidal streams where they construct permanent and semi-permanent galleries up to several meters deep into the anoxic, sulfide-rich sediment (Willis, 1942; Felder, 1978; Britton and Morton, 1989; Felder and Griffis, 1994; Felder, 2001). 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). The main objective of the present study is to evaluate a suite of physiological mechanisms that underlie the prolonged anoxia tolerance of this species.

Low resting 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* by Felder (1979) and *Neotrypaea californiensis* (formerly *Callinassa californiensis*) by Thompson and Pritchard (1969) and Torres et al. (1977) as well as other thalassinideans (see Stanzel and Finelli, 2004; Atkinson and Taylor, 2005). As ambient PO_2 approaches the respective P_{crit} value, crustaceans recruit fermentative pathways to provide additional ATP. Anaerobic glycolysis leading to the accumulation of lactate is probably the only source of ATP provision during 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 thalassinid shrimps survive anoxic conditions in the laboratory for several days (Thompson and Pritchard, 1969; Felder, 1979; Zebe, 1982). Though very small amounts of alanine, aspartate, glutamate, succinate, and malate have been reported in *N. californiensis*, L-lactate is the principal metabolic end product measured in that species and in *Upogebia pugettensis* (Pritchard and Eddy, 1979; Zebe, 1982). Unpublished observations suggest that lactate accumulates in *L. louisianensis* under hypoxia (Felder et al., 1995; Bourgeois and Felder, 2001).

Given that *L. louisianensis* survives anoxia nearly twice as long as *C. californiensis* and *U. pugettensis* (Felder, 1979), even at warmer temperatures, it is possible that anaerobic end products in addition to lactate are accumulated. Freshwater snails, mussels, oysters, and lugworms have anaerobically functioning mitochondria that accumulate succinate and the volatile fatty acids (VFAs) propionate and acetate via the malate dismutase pathway (see Grieshaber et al., 1994; van Hellemond et al., 1995; Tielens et al., 2002). Unlike aerobic mitochondria, which utilize ubiquinone for electron transfer to Complex III, the transfer of electrons to fumarate under anoxia is accomplished with rhodoquinone (van Hellemond et al., 1995; Tielens et al., 2002). Rhodoquinone is essential for anaerobic accumulation of succinate, propionate, acetate, and other VFAs including isovalerate, methylbutyrate, and isobutyrate (Lahoud et al., 1971; Hochachka, 1980; Holst and Zebe, 1986; Kita, 1992; Tielens, 1994). Thus we measured the amount of rhodoquinone present in the mitochondria of *L. louisianensis* to gain insight into the capacity for production of non-lactate end products.

Based on the strong positive correlation between the anoxia tolerance of species and their capacity for acute metabolic depression (Hand, 1998; Hochachka and Lutz, 2001), we predicted that under anoxia *L. louisianensis* would reduce metabolic rate severely and quickly (below 10% of the aerobic value), which would lower the rate of ATP consumption and conserve carbohydrate fuels. To estimate the degree of metabolic depression, we chose to estimate the rate of ATP turnover under anoxia from end product accumulation and arginine phosphate use, and then express the value as a percentage of the resting aerobic rate as calculated from oxygen consumption.

Such bioenergetic constraints under anoxia inevitably lead to ionic disturbances like calcium overload in cells, and the compromise of mitochondrial membrane potential ($\Delta\Psi$) will occur with extended time (Hochachka, 1986; Hand and Menze, 2008). When ATP availability drops so low that active ion transport across membranes cannot keep up with passive ion leak, then dissipation of ion gradients can take place (Covi and Hand, 2005; Covi et al., 2005; Covi and Hand, 2007). These phenomena signal initiation of apoptosis in mammals (Kroemer et al., 2007; Hand and Menze, 2008). Because of the remarkable tolerance to anoxia by *L. louisianensis*, we evaluated the susceptibility of mitochondria of this ghost shrimp to opening of the mitochondrial permeability transition pore (MPTP). If mammalian mitochondria are exposed to elevated calcium in the presence of phosphate, especially when accompanied by depletion of adenine nucleotides and reduced $\Delta\Psi$ across the inner membrane (Petronilli et al., 1993a), a large swelling occurs that is associated with uncoupled respiration and cytochrome-*c* release (Haworth and Hunter, 1979; Hunter and Haworth, 1979; Gunter and Pfeiffer, 1990; Halestrap et al., 2000; Kroemer et al., 2007). These phenomena are due to an acute increase in permeability of the inner mitochondrial membrane known as the permeability transition (Hunter et al., 1976; Bernardi, 1996; Bernardi et al., 2006;). Matrix swelling then causes the rupture of the outer mitochondrial membrane and release of numerous pro-apoptotic factors from the intermembrane space (Green and Reed 1998; Zamzami and Kroemer 2001; Green and Kroemer 2004; Saelens et al. 2004; Bernardi et al. 2006). Surprisingly, Menze et al. 2005b noted that the MPTP does not open in response to high calcium in mitochondria from another anoxia-tolerant crustacean, *Artemia franciscana*, so we hypothesized that the pore may be refractory to anoxia-induced activators in ghost shrimp. As proposed recently (Hand and Menze, 2008), a character trait like prolonged tolerance to anoxia may be a consequence, to some degree, of specific features of apoptosis operative across species. For example, a modest elevation in calcium may trigger apoptosis in mammals, whereas severe energy limitation may not initiate cell death in certain non-mammalian species. Functional trade-offs in the predisposition to environmental tolerance may have occurred in parallel with the evolution of diversified pathways for cell death in eukaryotic organisms.

2. Materials and Methods

2.1. 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 water pump ('yabby' pump). On occasion, the latter method was used when only small numbers of shrimp were needed.

Upon collection, shrimp were placed in perforated plastic vials to prevent aggressive interactions and transported to the lab in coolers 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 and those that contained roundworms (most commonly near the heart) were not used. Occasionally, non-ovigerous females were combined with males to obtain sufficient quantities of tissue for mitochondrial studies.

2.2. Quantification of anoxia tolerance

In preliminary experiments, we observed that small shrimp (<1 g wet wt.) survived substantially longer in anoxic seawater than large 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 we estimate that even the smallest animals used had a CL >10 mm). Thus all shrimp used were either sexually mature adults or within one molt from sexual maturity according to reproductive studies 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. 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 (about 98% of initial projections were accurate). 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⁻¹) or an antibiotic cocktail of 5 mg l⁻¹ chloramphenicol, 50 mg l⁻¹ gentamycin, 100,000 units l⁻¹ penicillin, and 100,000 µg l⁻¹ streptomycin was added to the seawater to test whether anoxia tolerance for adults of *L. louisianensis* could be extended by restricting growth of fungi or bacteria.

2.3. 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 N₂, and ground into a fine powder with a pre-chilled mortar and pestle under liquid N₂. The frozen powder (~2g) was then homogenized in a ground-glass homogenizer containing 5 volumes of ice-cold 6% perchloric acid (PCA) with 10 mmol l⁻¹ sodium ethylenediaminetetraacetic acid (EDTA). The acid-insoluble fraction was removed by centrifugation for 20 min at 10,000 g and 4° C. The supernatant was then neutralized with ice-cold 5 mol l⁻¹ K₂CO₃ and centrifuged at 10,000 g for 10 min and 4° 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. For adenylates, each PCA supernatant was divided into two equal aliquots. One aliquot was neutralized with ice-cold 5 mol l⁻¹ K₂CO₃ and the other with ice-cold 5 mol l⁻¹ K₂HPO₄, and then both were centrifuged as above to remove perchlorate salts. PCA extracts of tissues associated with molluscan and crustacean exoskeletons contain high Ca⁺⁺, which can cause significant precipitation of Ca⁺⁺-ATP and Ca⁺⁺-ADP upon neutralization of extracts with K₂CO₃ (cf. Rees and Hand, 1991), even with EDTA added to the PCA. Unpublished observations suggest that neutralization with K₂HPO₄ avoids this problem by preferentially precipitating the Ca⁺⁺ as CaPO₄ 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 -80°C until chemical analyses were performed. Upon thawing, any additional precipitate was removed by centrifugation.

2.4. 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_2O_2 is catalyzed by lactate oxidase. Using the H_2O_2 produced, peroxidase catalyzes the oxidative condensation of chromogen precursors producing a colored dye with an absorption maximum at 540 nm 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 mmol l^{-1} imidazole/HCl (pH 7.0), 2 mM Mg acetate, 10 mmol l^{-1} D-glucose, 0.5 mmol l^{-1} ADP, 1 mmol l^{-1} NADP, and 290 μl neutralized supernatant (diluted with deionized H_2O as needed). After baseline absorbance was recorded at 340 nm, 5 μl of hexokinase/glucose-6-phosphate dehydrogenase enzyme mixture (1.7 units HK, 0.85 units G6PDH; Roche Diagnostics, Mannheim, Germany) was added and the absorbance change recorded. Once all endogenous ATP was consumed, 5 μl of arginine kinase (AK; 10–20 mg protein ml^{-1} stock) 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), the functional properties of this crustacean enzyme having been described previously (Pruett et al., 2003).

2.5. 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 4°C in the auto sampler, and 75 μl of each sample were applied to a 250×4.6 mm strong anion exchange column (Sphereclone 5 μ SAX 80A, Phenomenex, Torrance, CA). The samples were eluted with a linear gradient from 40 mmol l^{-1} K_2HPO_4 (pH 5.5) to 500 mmol l^{-1} K_2HPO_4 (pH 5.5) over 26 min at a flow rate of 1 ml min^{-1} at 30°C . 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 $\mu\text{mol l}^{-1}$ to 1 mmol l^{-1} .

2.6. Isolation of mitochondria

Mitochondria were isolated from hepatopancreas tissue of shrimp using a protocol similar to Menze et al. (2005b). 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 mol l^{-1} sucrose, 150 mmol l^{-1} KCl, 1 mmol l^{-1} EGTA, 0.5% (wt/vol) fatty acid-free BSA, and 20 mmol l^{-1} K^+ -HEPES, pH 7.5]. The isosmotic pressure (750 mOsm) of isolation media for mitochondria of *L. louisianensis* was 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 4° 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 mol l⁻¹ sucrose, 150 mmol l⁻¹ KCl, 0.025 mmol l⁻¹ EGTA, 0.5% (wt/vol) fatty acid-free BSA, and 20 mmol l⁻¹ K⁺-HEPES, pH 7.5] and centrifuged again at 9,000 *g* and 4° 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 approximately 12–15 mg protein ml⁻¹. Protein was quantified using Coomassie Plus™ protein assay kit (Pierce, Rockford, IL) with BSA as the standard.

2.7. Mitochondrial volume change

Changes in volume of isolated mitochondria were measured spectrophotometrically as described in Menze et al. (2005b) and previously developed by (Petronilli et al., 1993b). A decrease in absorbance at 540 nm was indicative of an increase in mitochondrial volume (swelling). Measurements of mitochondria (0.8 mg protein/ml) were carried out at 25° C, and the reaction medium contained 300 mmol l⁻¹ sucrose, 150 mmol l⁻¹ KCl, 1 mmol l⁻¹ KH₂PO₄, 5 μmol l⁻¹ rotenone, 25 μmol l⁻¹ EGTA and 20 mmol l⁻¹ 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 mmol l⁻¹ succinate.

2.8. Fluorescence-based measurements of calcium

Calcium-induced fluorescence was measured according to Menze et al. (2005b). 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 mmol l⁻¹ 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 μmol l⁻¹ (Blattner et al., 2001), and fluorescence was excited from above the wells 20 min after addition of calcium. 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_{max}) obtained when the calcium probe was completely saturated with calcium in the respective buffer system. To obtain F_{max} the fluorescence was measured at intervals across a range of 1 to 1000 μmol l⁻¹ calcium. Data were fitted to the function: $F = (K \cdot X \cdot F_{\max}) / (1 + K \cdot X)$ 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_{max} is the maximal obtainable fluorescence.

2.9. Measurement of rhodoquinone:ubiquinone ratio

Isolated mitochondria were prepared as described above, except that the final wash and 9000 *g* centrifugation was 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 N₂, lyophilized, and sealed under N₂ 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 (van Hellemond et al., 1995; Hoffmeister et al., 2004).

2.10. Statistical analyses

GraphPad Prism Version 5.0 (GraphPad Software, San Diego, CA) was used for analysis of anoxia survival. The non-parametric Kaplan-Meier method was used to calculate median survival times (LT₅₀ values). A log-rank test (Mantel-Cox) was then used for comparing the two survival curves. SigmaStat 3.0 software (SPSS Inc., Chicago, IL) was used to perform all other statistical analyses. For lactate and arginine phosphate 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. For calcium-based fluorescence assays, simple t-tests were used.

3. Results

3.1. 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 at 25° C. The LT₅₀ for large shrimp (> 2 g) was 64.8 h (Figure 1) with one shrimp surviving 92 h without oxygen. In contrast, the LT₅₀ for small shrimp (<1g) was 113 h, which was nearly twice that of the large shrimp (Figure 1). The difference between survival curves for the two size groups was significant ($P < 0.0001$). 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). Addition of either an antibiotic cocktail (Materials and Methods) or a fungicide (amphotericin B) did not extend the survival of adults or juveniles (LT₅₀ of 62 and 100.5 h, respectively, using the antibiotic cocktail).

3.2. Metabolite levels during anoxia

Lactate accumulation in whole-animal extracts of *L. louisianensis* (large shrimp only) was negligible in the control group (Figure 2), but rose 18-fold in animals exposed to anoxia for 6 h. After 12 h of anoxia, lactate reached levels more than 40-fold those of control animals and more than 60-fold those of the controls after 24 h. Lactate concentration appeared to reach a plateau after 48 h ($118 \pm 17.0 \mu\text{moles g.f.w.}^{-1}$, mean $\pm$ SD, $N=6$) with no significant increase observed at 72 h of anoxia ($P > 0.05$, versus 48 h).

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 statistically-significant changes in the total pool of adenylates, ATP:ADP ratio, AMP:ATP ratio, or the AEC (Figure 3).

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 K₂CO₃, yet the same extract (equal volume derived from the same animal) neutralized with K₂HPO₄ contained substantially more ATP. This large difference was not observed for all extracts, but was sufficiently frequent that the adenylate data set collected with K₂CO₃ neutralization was discarded. The most likely reason for the large adenylate variability seen with K₂CO₃ 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.

The pool of arginine phosphate in *L. louisianensis* declined slowly across the anoxia time course (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 $3.78 \pm 0.93 \mu\text{moles g.f.w.}^{-1}$

by 48 h, which represents more than a 50% reduction from control levels ($8.22 \pm 0.45 \mu\text{moles g.f.w.}^{-1}$). The pool of arginine phosphate returned to control levels by 24 h post-anoxia.

Potential end-products of anaerobic metabolism other than lactate were not investigated primarily due to the finding that mitochondria of *L. louisianensis* do not contain physiologically-significant levels of rhodoquinone. The insignificant amount of rhodoquinone detected was less than 0.1% of the ubiquinone pool. 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. In such cases, rhodoquinone levels are at least 10% of the ubiquinone pool (Tielens et al., 2002).

3.3. Mitochondrial swelling and Ca^{2+} uptake

Swelling due to the opening of the MPTP can be observed as a decrease in the absorbance of mitochondria at 540 nm (Petronilli et al., 1993b). As shown in Figure 5, absorbance actually increases when 1 mmol l^{-1} calcium is added exogenously to energized mitochondria isolated from hepatopancreas tissue in *L. louisianensis*. 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., 2005b). The increase in absorbance observed is indicative of the transport of large quantities of calcium into the matrix (see Discussion). Addition of $20 \mu\text{mol l}^{-1} \text{HgCl}_2$, 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 adequate for detection.

A high capacity for calcium uptake was observed for energized mitochondria ($\sim 0.8 \text{ mg protein ml}^{-1}$) isolated from this anoxia-tolerant invertebrate. Because the fluo-5N dye cannot penetrate the mitochondrion, the fluorescence values measured are correlated 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. However, energized mitochondria significantly reduce the level of exogenously added calcium below the control at each concentration investigated. Above 0.2 mmol l^{-1} exogenous calcium, the free calcium levels begin to rise in energized mitochondrial preparations, but even at 1 mmol l^{-1} 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, there is a lack of calcium-induced *release* of calcium from the mitochondrial matrix in both energized and de-energized mitochondria.

4. Discussion

We have shown in this study that *L. louisianensis* has a remarkable tolerance to anoxia, and that it accumulates extremely high concentrations of lactate when exposed to chronic anoxia. To our knowledge, the lactate concentrations observed after 72 h of anoxia (over $125 \mu\text{mol g.f.w.}^{-1}$) are the highest ever reported for a crustacean species (cf. Zebe, 1982; Albert and Ellington, 1985; Taylor and Spicer, 1987; Hill et al., 1991; Anderson et al., 1994; Henry et al., 1994; Adamczewska and Morris, 2001; Burnett et al., 2006). Our data also suggest that this species may utilize, to a minor degree, arginine phosphate to buffer changes in ATP levels under anoxia. 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 (MO_2) of similar-sized animals by Felder (1979), we 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 downregulation of metabolism is delayed in *L. louisianensis* until after high levels of lactate are accumulated. This metabolic approach would support substantial physiological activities through 48 h. Behaviorally, for example, the delayed downregulation could permit periodic irrigation of the burrow as a way to detect the tidal return of high- PO_2 water outside the burrow. Locomotory movements to explore different regions of the gallery could be advantageous for similar reasons. Finally, calcium plus phosphate does not trigger opening of the regulated MPTP in mitochondria from *L. louisianensis* as it does in mammalian mitochondria. This result is significant in that it indicates this animal may be predisposed to extended anoxia tolerance. As postulated by Hand and Menze (2008), the evolution of diversified mechanisms for initiating cell death in mammals, for example, may have occurred in parallel with functional trade-offs in environmental tolerance.

4.1. Survival under anoxia

The mean survival time (LT_{50}) of *L. louisianensis* under anoxia in our study was 64.8 h for the large size class, which is slightly lower than the 3.2 days (~76 h) reported for similarly-sized animals of the same species under identical conditions by Felder (1979). This small difference is likely within the experimental error for the two studies. 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, specimens of *Calocaris macandreae* survive only 43 h at 10° C (Anderson et al., 1994). Specimens of *Neotrypaea californiensis* (formerly *Callinassa*) were originally reported to survive 138 h without oxygen at 10° C (Thompson and Pritchard, 1969), but later were found to survive only 52–60 h at 12° C (Zebe, 1982). The warmer habitats of *L. louisianensis* may experience more frequent and longer episodes of anoxia compared to the colder-water habitats. Whatever the case, no other decapods have been shown to survive such extended periods of anoxia in the laboratory.

The smaller size class of ghost shrimp (<1g) 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 do larger ones, and thus glycogen stores would presumably last longer during anaerobic 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 related to experimental conditions -- both size classes were exposed to anoxia in the same volume (300 ml) of water. One might argue that higher concentrations of waste products (e.g., protons, ammonia) accumulate in containers with the larger animals and reduce their survivorship. When Felder (1979) replaced anoxic water daily, the LT_{50} for *L. louisianensis* increased from 3.2 to 4 days.

Treatment of the clam *Macoma balthica* with chloramphenicol significantly increased the LT_{50} in anoxia from 4.8 to 13.3 days (de Zwaan et al., 2001). The same treatment extended the LT_{50} 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 LT_{50} for *L. louisianensis* in anoxia, nor did the addition of amphotericin B.

4.2. Lactate Generation

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 (cf. 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 *Calocaris macandreae* and *N. californiensis*; values reached $16.8 \pm 0.28 \mu\text{mol g.f.w.}^{-1}$ by 24 h in *N. californiensis* (Zebe, 1982) and $11.6 \mu\text{mol g.f.w.}^{-1}$ by 18 h in *C. macandreae* (Anderson et al., 1994). Comparatively, *L. louisianensis* produced nearly 3-fold more lactate ($65.2 \pm 9.6 \mu\text{mol g.f.w.}^{-1}$) by 24 h of anoxia, albeit at a much higher ambient temperature (Figure 2). By 48 h, the concentration in *L. louisianensis* nearly doubled ($118 \pm 17.0 \mu\text{mol g.f.w.}^{-1}$). 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.

4.3. Maintenance of adenylate pools and delayed metabolic depression

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 (cf. Table 2). Nevertheless, when combined, these two sources of ATP are sufficient to fully stabilize the adenylate pool during the 48 h bout of anoxia in tissues of *L. louisianensis*. There was a trend in the means for both ATP and the total adenylate pool to decrease slightly with exposure time, but the fairly large SEs precluded any statistical resolution of these patterns.

A novel and surprising insight is revealed from the calculations in Table 2, which suggest that during early stages of anoxia the sources of ATP above are sufficient to support a metabolic rate similar to that observed in normoxic-exposed animals at rest. 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 value increases 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 finally 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 is commonly observed in tolerant invertebrates, and the degree of depression is strongly correlated with anoxic survivorship (Hand and Hardewig, 1996; 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). However, such a downregulation is delayed in *L. louisianensis*.

4.4. Absence of a regulated MPTP

Our data demonstrate that 1 mmol l^{-1} 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, 2003; Nicholls and Chalmers, 2004). Addition of high concentrations of mercury ($20 \mu\text{mol l}^{-1}$) 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). The 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 mitochondria of *L. louisianensis*. In fact mitochondria of *L. louisianensis* continue to actively load calcium even when challenged with 1 mmol l^{-1} extra-mitochondrial calcium.

The lack of a calcium-induced permeability transition and the high calcium uptake capacity was also observed in *Artemia franciscana* by Menze et al. (2005b) 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 (Kroemer et al., 2007); the complex then can recruit and activate caspases. 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 is 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.

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Figure 1.

LT₅₀ (median survival times) of *L. louisianensis* under anoxia for two size classes. The LT₅₀ for large specimens (>2 g, N=22; closed circles) was 64 h and for small animals (< 1 g, N=17; open circles) was 113 h. The non-parametric Kaplan-Meier method was used to calculate LT₅₀ values. A log-rank test (Mantel-Cox) was used to compare the two survival curves, which are statistically different (P<0.0001). Error bars for the lines (± one S.E.) are included simply to indicate the degree of variance within the data sets.

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 symbol represents mean $\pm$ SD for n=6 experiments. (One-Way ANOVA, Tukey pair-wise comparison, $P < 0.001$).

Figure 3. Arginine phosphate concentration (bars) and adenylate energy charge (●) 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 $\pm$ 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.

Figure 4. Analysis of adenylate levels in *L. louisianensis*. Representative chromatograms from neutralization of a perchloric acid extract from the same animal with K_2HPO_4 (upper frame) and K_2CO_3 (lower frame). Neutralization with K_2HPO_4 fosters substantially higher recovery of ATP. See text for further explanation.

Figure 5.

Effects of Ca^{2+} and HgCl_2 in the presence of 1 mmol l^{-1} 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 mmol l^{-1} CaCl_2 to energized mitochondria ($0.8 \text{ mg protein ml}^{-1}$ in 300 mmol l^{-1} sucrose, 150 mmol l^{-1} KCl, 1 mmol l^{-1} KH_2PO_4 , 5 μmol l^{-1} rotenone, 25 μmol l^{-1} EGTA, and 20 mmol l^{-1} K-HEPES, pH 7.5). Mitochondrial volume increases after addition of HgCl_2 .

Figure 6.

Ca^{2+} uptake by isolated mitochondria from hepatopancreas of *L. louisianensis*. Mitochondria (~ 0.8 mg protein ml^{-1}) were incubated in exogenously added Ca^{2+} in the presence of the Ca^{2+} probe fluo-5N, which measures the extra-mitochondrial Ca^{2+} . Fluorescence was expressed as a percentage of the total fluorescence when the dye is saturated with Ca^{2+} (% F_{max}). Control samples contained no mitochondria. Energized mitochondria (succinate plus rotenone) reduced the concentration of exogenously added Ca^{2+} at all concentrations investigated, relative to controls. De-energized mitochondria (no succinate; with either rotenone or FCCP) were less effective in calcium uptake than energized mitochondria. Each bar represents mean $\pm$ SD of N=4 experiments. *Significantly different from control (ANOVA, Tukey pairwise comparison, $P < 0.001$)

Table 1

Adenylylate levels in *L. louisianensis* exposed to a time course of anoxia.^a

	Control	Hour 6	Hour 12	Hour 24	Hour 48	Recovery
AMP	0.63 ± 0.16	1.13 ± 1.46	0.59 ± 0.15	0.69 ± 0.19	1.40 ± 0.82	0.86 ± 0.45
ADP	7.85 ± 1.51	9.83 ± 3.07	7.66 ± 0.42	8.10 ± 0.77	9.05 ± 1.34	6.49 ± 0.32
ATP	73.64 ± 5.26	67.38 ± 6.20	63.28 ± 10.42	59.57 ± 13.19	56.32 ± 2.57	60.29 ± 4.95
SUM	82.12 ± 5.19	78.34 ± 10.48	71.53 ± 10.95	68.36 ± 12.56	66.77 ± 2.56	68.04 ± 5.31
ATP:ADP	9.60 ± 1.83	7.15 ± 1.44	8.23 ± 0.95	7.46 ± 2.16	6.32 ± 1.02	9.35 ± 0.39
AMP:ATP	0.01 ± 0.00	0.02 ± 0.02	0.01 ± 0.00	0.01 ± 0.01	0.03 ± 0.02	0.01 ± 0.01

^aThe recovery group was given 24 h of normoxia after the 48 h anoxic exposure. Values are reported in $\mu\text{mol g.f.w.}^{-1}$ (whole body). Means $\pm$ one standard deviation (N=3) are given for all treatment groups. No significant differences were observed across experimental treatments for any adenylylate value; the P values for comparisons of means were all greater than 1.48 (One-Way ANOVA).

Table 2

Percentage of resting aerobic metabolism maintained under anoxia in *L. louisianensis* calculated as the ATP yields from lactate and arginine phosphate.^a

Time in anoxia (h)	Lactate produced	ATP yield from lactate	Arg-P used	ATP yield from Arg-P	Total ATP yield	ATP yield via aerobic respiration	Percentage of aerobic rate maintained under anoxia
0-6	18.4	36.8	1.8	1.8	38.6	72.9-53.5	53-72
6-12	26.2	52.5	2.0	2.0	54.5	72.9-53.5	75-100
12-24	20.6	41.1	0.5	0.5	41.6	145.8-107.0	29-39
24-48	53.3	106.6	0.1	0.1	106.7	291.6-214.0	36-49
48-72	3.8	7.6	0.0	0.0	7.6	291.6-214.0	3-4

^aUnits for lactate, ATP and arginine phosphate are $\mu\text{mol g.f.w.}^{-1}$ produced or consumed across the indicated time intervals. For comparison, ATP yield from resting aerobic respiration is calculated from the data of Felder (1979), which gives respiration rates between 50 and 68 $\mu\text{l O}_2 \text{ h}^{-1} \text{ g.f.w.}^{-1}$ at 25° C for animals ranging from 1.45 to 7.13 g. Rates were converted to $\mu\text{mol O}_2 \text{ h}^{-1} \text{ g.f.w.}^{-1}$, and then a P:O ratio of 2 was used to calculate ATP synthesis [average value for Complex I (1.5) and Complex II (2.5); Nichols and Ferguson, 2002]. Animals in the present study were incubated at 25° C, and sizes ranged from 2 to 5 g.