Lactate Dehydrogenase and Na^+/K^+ ATPase Activity in *Leiostomus xanthurus* (Spot) in Response to Hypoxia

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ABSTRACT

The purpose of this experiment was to study the effects of hypoxic environments on two main enzymes in Leiostomus xanthurus (spot). The spot were placed in containers containing low dissolved oxygen contents for varying time periods. Soon after, tissue samples were taken to study lactate dehydrogenase (LDH) and sodium-potassium ATPase (i.e, sodium potassium pump) activity. Our results showed quite a bit of inter-individual variation among samples. Regarding LDH activity, enzyme activity appeared to increase initially in muscle tissue, but it did not remain elevated over time. In gill tissue, LDH activity decreased over time, possibly due to a shut-off of metabolic activity after low oxygen exposure. ATPase activity also showed an increase in activity in liver tissue, but like with LDH activity in muscle, this did not maintain over time. Overall, this experiment was successful in studying the effects of hypoxia on enzyme activity in spot, however, more experiments should be conducted in order to decrease the inter-individual variation we found.

INTRODUCTION

Aquatic environments can pose a variety of stresses to resident organisms. A major stress that aquatic organisms often encounter is hypoxia, or a reduced dissolved oxygen (DO) concentration. A hypoxic environment is defined as one containing less than 2 mg/L DO (1). Estuarine fish who live in the regions between rivers and oceans encounter the lowest dissolved oxygen concentrations when temperatures are high (during the summer) and at night-time. Given that hypoxia is a major stress to estuarine organisms, we are interested in how it affects their physiology, specifically metabolic respiration. We chose to look at spot, Leiostomus xanthurus, because like many estuarine organisms, they are moderately hypoxia-tolerant (1).

Anaerobic respiration can be measured by an enzyme known as lactate dehydrogenase (LDH). LDH catalyzes the conversion of pyruvate (the product of glycolysis) to lactate with concomitant conversion of NADH to NAD^+ (nicotinamide adenine dinucleotide), which can be measured spectrophotometrically. It is therefore hypothesized that lactate dehydrogenase activity will increase after prolonged exposure to hypoxic conditions. In fact, one study performed in vascular smooth muscle cells of rats concluded that cells exposed to hypoxia

for 24 hours had a two-fold increase in LDH activity (2). Another study performed in liver tissue of the killfish Fundulus heteroclitus also concluded a two-fold increase in LDH activity, this time after two days of hypoxia (3). In this study we report an initial increase in LDH activity, but this increase is inconsistent over time.

Adenosine triphosphate (ATP), the major energy currency of the cell, is hydrolyzed by Na^+/K^+ ATPase, otherwise known as the sodium potassium pump. This membrane-bound enzyme uses energy from ATP hydrolysis to pump three Na^+ ions out of the cell and two K^+ ions into the cell. These gradients are necessary for sustaining membrane potential, cell volume, and secondary transport of solutes. The Na^+/K^+ pump alone accounts for approximately 30% of ATP consumption in the cell (4-6). It was therefore hypothesized that the activity of this pump is decreased with prolonged exposure to hypoxic conditions to preserve ATP concentrations within the cell. Using ouabain-sensitive Na^+/K^+ ATPase, the absorbance of p-nitrophenol produced from the reaction of the ATPase with p-nitrophenylphosphate was used as indicator of ATPase activity. Previous experiments have shown the activity of Na^+/K^+ ATPase to decrease after acute deoxygenation, particularly in hepatocytes (liver cells) and alveolar epithelial cells (4), while others show only a decrease in Na^+/K^+ ATPase activity in alveolar epithelial cells after prolonged exposure with no response to acute hypoxia (6). In this experiment, we report an initial increase in Na^+/K^+ ATPase activity after 24 hours of exposure, followed by a decrease at 48 and 72 hours in liver samples.

PROCEDURE

For each of three experiments, eight eleven-liter tanks were filled with water containing Instant Ocean® to 5.7% salinity. A mixture of nitrogen and air was introduced into six of the tanks via a gas mixer to a dissolved oxygen concentration of ~2 mg/L or 25% oxygen saturation. These tanks were to serve as experimental tanks. Air was introduced into two of the tanks to a concentration of 8-9 mg/L oxygen (100% saturation) to serve as control tanks. Three fish were placed in each of the six experimental tanks. Sizes and weights were similar between fish used. One fish from each tank was removed at periods of 24, 48, and 72 hours, dissected, and gill, muscle, and liver tissue removed for homogenization and assay analysis. Three fish were placed in each of two control tanks and removed at the same time periods. In addition, six fish were removed from the holding tank at time zero.

Tissues were suspended in 50 mM Tris-HCl buffer with 0.1% Triton, 0.1mM phenylmetylsulfonyl fluoride (PMSF) and 0.5mM EDTA (pH 7.5). The tissues were placed in homogenization tubes and homogenized on ice with a Virtishear Cyclone I.Q. homogenizer. After homogenization, the samples were centrifuged in an Eppendorf microcentrifuge at 11,250 revolutions per minute for thirty minutes and then a protein dilution of each supernatant was made. Supernatant and protein dilutions were then frozen at -80°C for future use.

For LDH analysis, homogenized muscle and gill samples were thawed and centrifuged for five minutes to clear. Ten-fold dilutions of muscle and five-fold dilutions of gill were made in 50 mM potassium phosphate buffer pH 7, 2.5 mM EDTA and kept on ice. A small amount of homogenate (between 5-20 µL) was combined with 0.4 milliliters of 50 mM imidazole/1.0 mM potassium cyanide (pH 7.5) along with 0.5 milliliters of 50 mM Imidazole/0.15 mM NADH. Absorbance was recorded in triplicate to serve as the control. Next, 0.1 milliliters of 5 mM pyruvate was added to each solution and change in absorbance over a

one minute time frame was immediately recorded. This rate was then converted to LDH activity by multiplying by a factor of -0.1660 based on the extinction coefficient of NADH (7). Dilutions and sample volume were then used to determine LDH activity per milliliter of sample. Protein activity of each tissue sample, which was previously calculated, was then used to find the LDH activity per milligram protein.

For Na^+/K^+ ATPase analysis, homogenized liver samples were thawed and centrifuged for five minutes at 13,000 rpm to settle fat from the samples. Five-fold dilutions of liver were made in 1.0 M Tris-HCl buffer pH 7.5, and kept on ice. The assay consisted of two solutions, A and B. Solution A, which contained 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 10 mM p-nitrophenylphosphate (p-NPP), and 25 mM KCl was used to determine the total ATPase. Solution B, which contained 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 10 mM p-NPP, and 3 mM ouabain was used to measure total ATPase minus Na^+/K^+ ATPase. Twenty microliters of the diluted liver homogenate were pipetted into the appropriate wells in a Microlite® plate, followed by 120 microliters of solution A or B. The reaction between each homogenate sample and each solution was carried out in triplicate. After incubating at 25°C for ten minutes, the reactions were stopped by the addition of 80 microliters of 1 M sodium hydroxide (NaOH). The absorbencies of the samples were then taken using a SpectraMAX 250 microplate spectrophotometer running SOFT MAX Pro 2.1 Alias software. Blank NaOH absorbance was subtracted from the total absorbance to give absorbance of p-nitrophenol. The formation of p-nitrophenol from p-NPP during Na^+/K^+ ATPase activity was the basis for absorbance. The ouabain in solution B inhibited the reaction. Therefore, the p-nitrophenol absorbance of the reactions with solution B were subtracted from the p-nitrophenol absorbance of Solution A, giving the Na^+/K^+ ATPase activity reported in nmoles min^{-1} mg^{-1} of protein. The absorption coefficient of p-nitrophenol is 13,200 M^{-1} cm^{-1}.

RESULTS AND DISCUSSION

LDH Analysis in Muscle and Gill

When oxygen supply is low, many organisms switch from aerobic metabolism to anaerobic metabolism in order to maintain their function. Because LDH is an enzyme used under anaerobic conditions, we expected that the LDH activity in spot would increase after being exposed to hypoxic conditions. Data were evaluated using ANOVA statistical analysis and graphs are shown below. The exposed fish can be compared to the time-zero fish. It appears that LDH activity initially increases after 24 hours and then decreases after 48 and 72 hours (shown in panels A and C). We concluded that spot exposed to hypoxia show an initial increase in the activity of LDH (and possibly other enzymes) in muscle tissue, but activity level does not remain elevated after 48 hours.

Panels D through F show the average LDH activity occurring in the gill tissue of spot. The LDH activity in gill tissue is significantly lower than the activity in muscle tissue (approximately 1/10 the activity). In panels D and E, it appears that LDH activity decreases over time, specifically after 24 and 48 hours. One hypothesis is that there is a shut-down of specific metabolic activities in gill after hypoxia exposure.

Due to a large amount of inter-individual variation among the spot, we were unable to come to a significant conclusion regarding LDH activity in muscle and gill tissue of spot.

LDH Analysis in Pooled Muscle Tissue

Muscle tissues samples were pooled for analysis. In the pooled tissue analysis, we

expected an increase in LDH activity as we expected in the muscle and gill tissue. We hypothesized that the pooling of samples would decrease the inter-individual variation originally present. Panels G-I show the average LDH activity occurring in the pooled muscle samples of spot that have experienced hypoxia for various time intervals. In Experiment 1 and Experiment 3, it is very evident that the amount of LDH activity in fish exposed to hypoxia is much greater than that of time zero fish. In addition, control fish do not change over time. However, because the individual samples were pooled, statistical analysis could not be carried out to determine any significant differences.

Panel J shows the average of pooled sample data of Experiments 1 through 3 in muscle tissue. The LDH activity of the time zero fish and control fish seems to remain at a constant level of approximately 3 units/mg protein. The fish exposed to hypoxia, however, have an average activity of close to 6 units/mg protein after 24 hours of exposure. The LDH activity in fish exposed to hypoxia are almost double that of fish who experienced no hypoxia. Error bars are not shown in this graph because n=3, therefore any trends of significant difference are not able to be determined. Therefore, it can be suggested that hypoxic conditions increase anaerobic respiration in spot, but more data need to be gathered to confirm these results.

Lastly, the pooling of samples seems to decrease the effect of inter-individual variation. As shown in panel J, the control and time zero fish seem to remain at a constant LDH activity level. The fish that experienced hypoxic conditions for either 24, 48, or 72 hours also seem to remain at a constant LDH activity level. This suggests that the pooling of samples decreases the variation originally present in individual samples.

ATPase Analysis in Liver

Hypoxia-tolerant organisms typically down regulate their major ATP consuming pathways when presence of oxygen is low. Na^+/K^+ ATPase uses a large amount of ATP, and it was expected that spot would down regulate this pathway after being exposed to hypoxia. The results that we found did not match those that were expected. Panels K through M show the average ATPase activity occurring in the liver tissue of spot. In all three experiments, it appears as if the ATPase activity initially increases (after 24 hours) from time zero, and then drops after 48 or 72 hours. This initial increase, which also occurred with the LDH activity in muscle, may be the result of an increase in enzyme activity after being exposed to hypoxia, but the increase was not maintained after 48 or 72 hours.

Overall, this experiment was successful in comparing both LDH activity and ATPase activity in various tissues of spot that were exposed varying levels of hypoxia. Future experiments are needed in order to determine more significant differences between tissue samples.

REFERENCES

1. Ross, SW, DA Dalton, S Kramer, and BL Christenson. 2001. Physiological (antioxidant) responses of estuarine fishes to variability in dissolved oxygen. Comp Biochem Physiol C Toxicol Pharmacol 130(3): 289-303.

2. Marti, H, H. Jung, J. Pfeilschifter, and C. Bauer. 1994. Hypoxia and cobalt stimulate lactate dehydrogenase (LDH) activity in vascular smooth muscle cells. Pflugers Arch 429:216-222.

3. Morton, J. 1995. Short term response of LDS activity to hypoxia in Fundulus herteroclitius. Hopkins Marine Station Student paper

4. Bogdanova, A., B. Grenacher, M. Nikinmaa, and M. Gassmann. 2005. Hypoxic responses of Na^+/K^+ ATPase in trout hepatocytes. J Exp Biol 208:1793-1801.

5. Comellas, A. P., L. A. Dada, E. Lecuona, L. M. Pesce, N. S. Chandel, N. Quesada, G. R. Budinger, G. J. Strous, A. Ciechanover, and J. I. Sznajder. 2006. Hypoxia-mediated degradation of Na,K-ATPase via mitochondrial reactive oxygen species and the ubiquitin-conjugating system. Circ Res 98:1314-1322.

6. Dada, L. A., N. S. Chandel, K. M. Ridge, C. Pedemonte, A. M. Bertorello, and J. I. Sznajder. 2003. Hypoxia-induced endocytosis of Na,K-ATPase in alveolar epithelial cells is mediated by mitochondrial reactive oxygen species and PKC-zeta. J Clin Invest 111:1057-1064.

7. Hansen, C. A. and B. D. Sidell. 1983. Atlantic hagfish cardiac muscle: metabolic basis of tolerance to anoxia. Am. J. Physiol. Regul. Interg Comp Physiol 244: R356-362.

GRAPHS

Graphs shown below are separated by tissue analysis (muscle, gill, liver) as well as enzyme studied (LDH versus ATPase). Letters above the bars represent statistically significant differences (unlike letters, such as a and A) or no significant differences (similar letters, such as A and A).
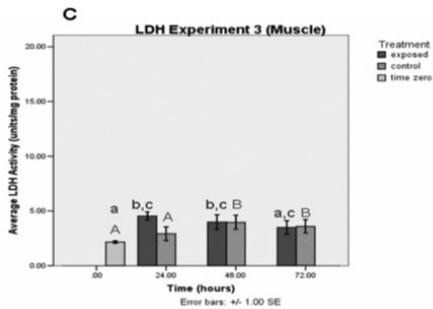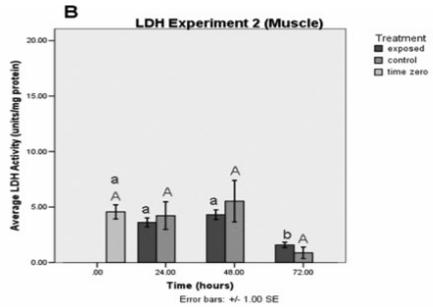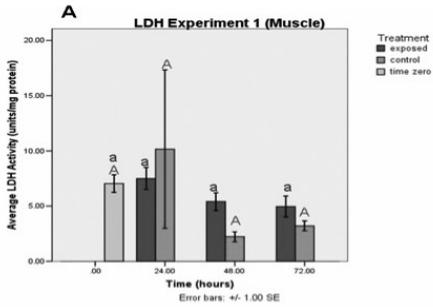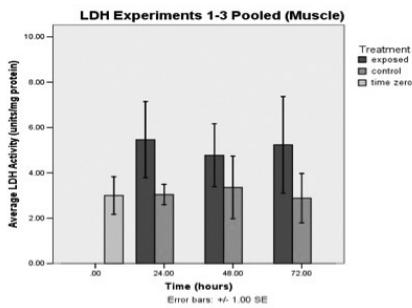

Panels A-C: Average LDH activity occurring in muscle tissue in exposed, control, and time-zero fish
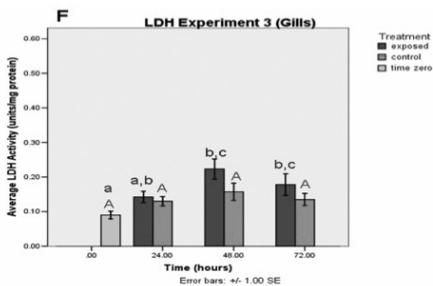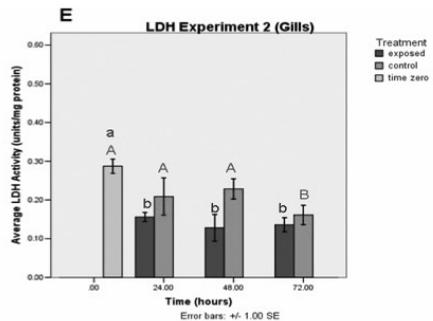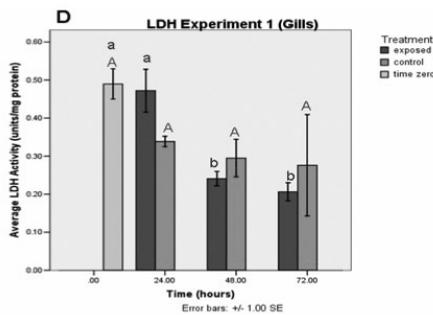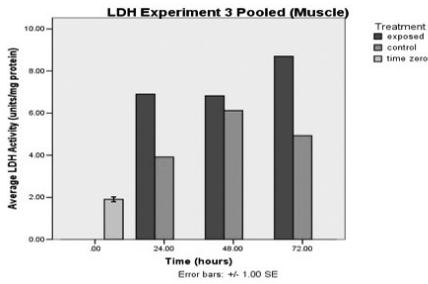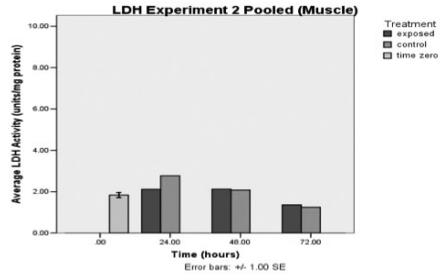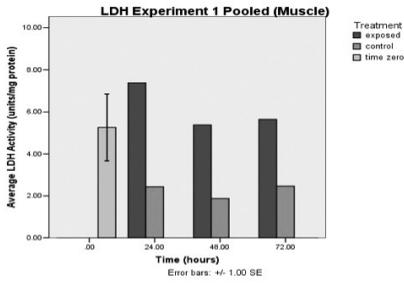

Panels D-F Average LDH activity occurring in gill tissue in exposed, control, and time-zero fish (Exp 1)
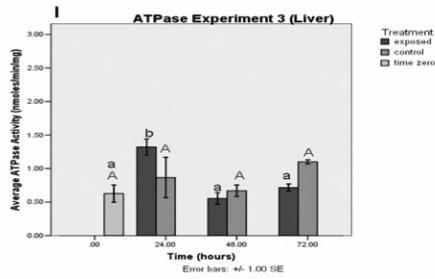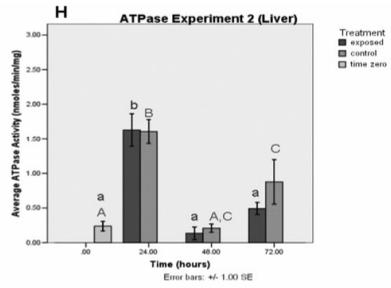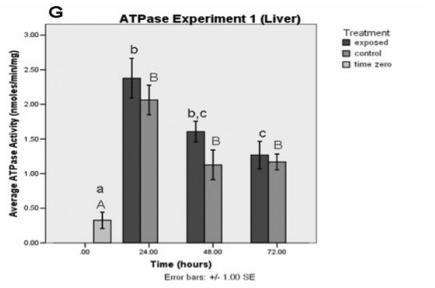
Panels G-I. Average ATPase activity occurring in liver samples of spot that have experienced hypoxia for various time intervals.

Panel J. Average LDH activity occurring in pooled muscle samples (Exp 1-3) of spot that have experienced hypoxia for various time intervals.

LDH Experiment 1 Pooled (Muscle)

LDH Experiment 2 Pooled (Muscle)

LDH Experiment 3 Pooled (Muscle)

Panels K-M. Average LDH activity occurring in pooled muscle samples of spot that have experienced hypoxia for various time intervals.