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Metabolic Support Of Anaerobiosis In Embryos Of The Annual Killifish

Austrofundulus Limnaeus

by

Andrew McCracken

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biology

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Portland State University
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Abstract

Embryos of the annual killifish *Austrofundulus limnaeus* display a remarkable tolerance to anoxia during development, most notably during embryonic diapause. Little is known about the metabolic or enzymatic changes that accompany this state of anoxia tolerance. This study examined the metabolic changes associated with exposure to anoxia by measuring the activity of the enzyme phosphoenolpyruvate carboxykinase (PEPCK), and by profiling the concentration of 31 metabolites ranging from amino acids to citric cycle intermediates at 4 different developmental stages, diapause 2 (DII), 4 days post diapause (dpd), 12 and 22 dpd. Embryos of *A. limnaeus* showed stage specific changes in concentrations of several metabolites. The most notable changes in metabolite concentration in response to anoxia were the increases of lactate, alanine, GABA and succinate as well as a pronounced decrease in aspartate concentrations. However, a complete understanding of the mechanisms by which anoxia tolerance is achieved remains elusive. Further studies into the tissue specific responses of anoxia would enable greater resolution when attempting to explain changes in concentrations of metabolites both during development and in response to anoxic insult.

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Introduction

Oxygen is perhaps the most important molecule in regards to sustained metabolic activity in vertebrate tissues. Vertebrate brains show a pronounced sensitivity to a lack of oxygen and are unable to maintain normal ATP levels after only minutes when blood perfusion is blocked (ischemia) (Michenfelder et al. 1971). The inability to maintain ATP levels in neuronal cells leads to the activation of a number of signaling cascades that ultimately result in cell death (Lipton 1999). While most mammalian cells are incapable of surviving extended periods of time without oxygen, there are some non-mammalian vertebrate species that have evolved methods of dealing with this environmental variability rather effectively. For example, North American freshwater turtles and the crucian carp have evolved remarkable tolerances to anoxia that allow survival in their highly variable environments (Blazka 1958, Bickler 1992, Johansson et al. 1995, Johansson et al. 1995, Hochachka et al. 1997, Bickler et al. 1998, Jackson 2000, Jackson 2002, Nilsson et al. 2004, Dinkelacker et al. 2005). Freshwater turtles utilize severe metabolic depression to survive anoxia while crucian carp have been shown to maintain modest levels of metabolic activity through the production of ethanol to avoid toxicity commonly associated with glycolytic end products such as lactate (Nilsson et al. 2004). In this study we use the unique biology of the annual killifish *Austrofundulus limnaeus* to investigate the metabolic underpinnings of extreme anoxia tolerance in a vertebrate species that exhibits a wide range of developmental stage-specific tolerances to anoxia.

Annual Killifish

The annual killifish, *Austrofundulus limnaeus*, is native to the Maracaibo basin, in northern Venezuela. This region experiences varying extremes in climate including a distinct dry and rainy season. *A. limnaeus* inhabits ephemeral ponds in coastal desert and tropical savanna regions; these ponds are often isolated from permanent water by several km of distance. The ponds in which *A. limnaeus* was found showed extreme diurnal variation in oxygen content and temperature, as well as high interpool variation in temperature, oxygen content, pH and ion composition (Podrabsky et al. 1998). Embryos of *A. limnaeus* are deposited into anoxic or extremely hypoxic sediments at the bottom of the rainy season pools (Podrabsky et al. 1998). With the onset of the dry season the ponds dry killing the adult and juvenile fish. Populations may persist in a given location due solely to the survival of embryos buried in the drying mud.

Diapause: Embryos of *A. limnaeus* can enter a state of dormancy called diapause in which they possess the ability to survive extended periods of environmental stress (Wourms 1972, Podrabsky et al. 1999, Podrabsky et al. 2001, Machado et al. 2007, Podrabsky et al. 2007, Podrabsky et al. 2010, Podrabsky et al. 2012). Diapause is a developmentally programmed, endogenously triggered, and pre-emptive form of dormancy, and as such embryos enter diapause under conditions that are conducive for development (Hand 1998). In addition, embryos can also enter a state of dormancy known as quiescence in response to specific environmental cues, such as lack of oxygen (Podrabsky et al. 2012). Prior to hatching, *A. limnaeus* embryos may enter up to three distinct stages of diapause,

termed diapause I, II, and III; the most stress-tolerant stage being diapause II (Wourms 1972a, Wourms 1972b, Wourms 1972c, Podrabsky et al. 2001, Machado et al. 2007, Podrabsky et al. 2007).

Diapause I: Diapause I may occur during early development and is associated with a unique developmental process in annual killifish embryos termed dispersion and reaggregation of the embryonic blastomeres (Wourms 1972b, Wourms 1972, Wourms 1972). Diapause I is often induced in response to environmental stress, especially low oxygen or reduced temperatures (Wourms 1972c). Embryos of the laboratory stock of *Austrofundulus limnaeus* at Portland State University do not routinely enter diapause I but continue normal development when incubated under aerobic conditions (Podrabsky et al. 1999).

Diapause II: Embryos of the PSU laboratory stock of *A. limnaeus* regularly enter diapause II at 24 days post-fertilization. Diapause II is associated with a gain of tolerance to environmental stressors (Podrabsky et al. 2001, Machado et al. 2007, Podrabsky et al. 2007) and is characterized by a profound metabolic depression, a decrease in oxygen consumption, as well as a decrease in heart rate (Podrabsky et al. 1999). A small proportion (10-20%) of embryos do not enter diapause II and proceed directly toward hatching when incubated at 25°C under laboratory conditions (Podrabsky et al. 2010). These embryos display a different metabolic trajectory and enzymatic capacities than their counterparts who have undergone diapause (Chennault et al. 2010). Embryos can survive in diapause II for several months (perhaps even years) and resume development when exposed to longer photoperiods and increased temperatures (Podrabsky et al. 1999).

Diapause III. Once embryos exit diapause II, they initiate the major phases of developmental organogenesis, complete development and either hatch or enter diapause III. Most embryos (greater than 90%) enter diapause III. Diapause III is another state of metabolic and developmental arrest that occurs in the late pre-hatching embryos (Wourms 1972, Wourms 1972, Podrabsky et al. 1999). Diapause III embryos appear ready to hatch at any time environmental conditions may prove favorable.

Developmental Progression and Tolerance of Environmental Stress

Unlike most other vertebrates, *A. limnaeus* embryos possess the unique ability to withstand extended periods of what may be considered “stressful” conditions during embryonic development such as desiccation, high salinity, dehydration, and anoxia (Myers 1952, Podrabsky et al. 2001, Machado et al. 2007, Podrabsky et al. 2007). Tolerance of environmental stress is gained during early development, peaks during diapause II, and is gradually lost after the embryo exits diapause II and develops toward hatching. This study examines the metabolic response of *A. limnaeus* embryos to extended periods of anoxia as extreme tolerance of anoxia is lost during post-diapause II development.

Anaerobic Metabolism in Fish

Cellular ATP production can be categorized into two general classes, those reactions that require oxygen such as mitochondrial oxidative phosphorylation, and those reactions that do not such as glycolysis (Wilde et al. 1963). Fish brains have

been shown to have similar energy demands as mammals when temperature is taken into account (Nilsson et al. 2004). When compared to less tolerant species, anoxia tolerant species of fish exhibit a decreased energy demand accompanied by a greater increase of inhibitory neurotransmitters such as GABA and glycine and a decrease in glutamate when exposed to anoxia (van Ginneken et al. 1996). Teleosts that are more anoxia tolerant can also increase the use of anaerobic glycolysis and/or enhance the mobilization of glycogen (DiAngelo et al. 1988), which correlates with the finding that teleosts increase blood glucose levels during anoxia (Lutz et al. 1997).

The Role of Phosphoenolpyruvate Carboxykinase in Anaerobic Metabolism

Phosphoenolpyruvate carboxykinase (PEPCK) is an enzyme that is considered as the rate limiting step in gluconeogenesis from lactate (Rognstad 1979) and is found in several tissues in vertebrates including liver, brain, muscle and adipose tissue (Zimmer et al. 1990). PEPCK catalyzes the reaction between oxaloacetate and phosphoenolpyruvate (PEP) (Jomain-Baum et al. 1978). From PEP, the glycolytic carbon chain can then be converted to pyruvate via the enzyme pyruvate kinase and used in the citric acid cycle to create energy in normoxicaerobic conditions (Storey 1993). PEP can also be used to create glucose through gluconeogenesis or used to create glycerol-3-phosphate through glyceroneogenesis. Under aerobic conditions, the activity of PEPCK is maintained at relatively low levels, as the majority of the flux through the glycolytic pathway is dependent on high pyruvate kinase activity to produce pyruvate from PEP (See Fig. 1).

Figure 1. Metabolic pathway of PEPCK under aerobic conditions.

In marine mollusks, it has been shown that anoxia causes a change in concentration of metabolites and activity of key metabolic enzymes (an increase in alanine and phosphorylation of pyruvate kinase) to the point where pyruvate kinase activity is non-existent and the resulting glycolytic flux is rerouted through PEPCK in order to maintain succinate synthesis (Storey 1993).

Figure 2. Metabolic pathway of PEPCK under anoxic conditions.

PEPCK can be upregulated to accommodate any necessary changes in metabolism, i.e. when glucose is needed, or downregulated when glucose is not limiting. Hypoxia (Choi et al. 2005), glucagon, glucocorticoids, thyroid hormone, epinephrine and cAMP have been shown to increase the transcription of the gene for PEPCK (Lamers et al. 1982) while insulin and glucose have been shown to inhibit its transcription (Sasaki et al. 1984).

Metabolism of Anoxic A. limnaeus embryos

Embryos of *A. limnaeus* can survive for months in the complete absence of oxygen at temperatures between 20 and 30°C (Podrabsky et al. 2007, Podrabsky et al. 2012). This tolerance is several orders of magnitude greater than typical vertebrates and at least one order of magnitude greater than what are classically considered anoxia tolerant vertebrates (Podrabsky et al. 2012). Past studies of anoxia tolerance of *A. limnaeus* have focused primarily on early embryos from fertilization through diapause II, a time period when extreme anoxia tolerance is gained in association with entrance into diapause II (Podrabsky et al. 2007). In early embryos, extreme tolerance of anoxia is supported by a profound metabolic depression (Podrabsky et al. 2012). Surprisingly, early embryos through diapause II exhibit anaerobic metabolism fairly typical of average vertebrates, and accumulate large quantities of lactate, with smaller contributions from the accumulation of succinate, alanine, and GABA (Podrabsky et al. 2007). Even more surprisingly, anoxic embryos experience an 80% decline in levels of ATP, without the negative consequences associated with low ATP levels in non-tolerant vertebrates

(Pergusson-Kolmes et al. 2007, Podrabsky et al. 2007, Podrabsky et al. 2012). Of special interest is the ability of anoxia tolerant embryos to accumulate of 5-10 mM quantities of γ -amino butyric acid (GABA)(Podrabsky et al. 2007). In other anoxia tolerant vertebrates, GABA is accumulated in brain tissue in response to anoxia, but only in micromolar quantities; this accumulation is thought to help reduce the activity of neurons through GABA's role as an inhibitory neurotransmitter in the adult vertebrate brain (Lutz 1992, Lutz et al. 2004, Lutz et al. 2004).

In this thesis I further explore the metabolic response of *A. limnaeus* embryos to extended periods of anoxia by measuring the enzymatic activity of PEPCK as well as the concentration of 30 different metabolites. The 30 metabolites, arranged by functional group, are; anaerobic metabolites lactate, alanine and GABA; citric acid cycle intermediates succinate, malate, fumarate, citrate, c-aconitate and alpha ketoglutarate; essential and non-essential amino acids; citric acid cycle family amino acids like aspartic acid and asparagine; and other metabolites such as ascorbate, phosphate and glycerol-3-phosphate.

The goal of this study is to better characterize the metabolic processes and pathways that are associated with extreme tolerance of anoxia in early post-diapause II embryos and identify the changes in metabolic pathways associated with loss of anoxia tolerance during post-diapause II development in embryos of *A. limnaeus*. I hypothesize that embryos will show an increase in PEPCK activity and GABA concentrations when exposed to anoxia and then decrease during aerobic recovery from anoxia. I also hypothesize that post-diapause embryos will show

increased concentrations of lactate, succinate and alanine as well as a possible decrease in concentration of citric acid cycle metabolites in response to anoxia.

Materials and Methods

Animal Care and Facilities

Adult killifish were originally obtained from the Maracaibo Basin, Venezuela in 1995 and are currently housed in the Aquatic Vertebrate Facility at Portland State University. All fish care was performed in accordance with practices described in Podrabsky (1999). Water was changed twice daily (20% of total system volume) and the fish were fed Chironomid larva (frozen blood worms, Hikari, Hayward, CA) or earthworms twice daily. Water temperature was controlled at $26 \pm 1^\circ\text{C}$ and the light schedule was 14 hours of light and 10 hours of dark. Forty-two spawning pairs of adult fish were housed in 10-l glass aquaria (one pair per aquarium) with a plastic divider separating the two fish. Fish were allowed to spawn twice a week by removing the divider and placing a container of 500 μm glass beads into the aquarium. The resulting embryos were incubated at 25°C in embryo media that was formulated to be similar to natural killifish pond water in the Maracaibo basin (Podrabsky 1999). For the first 4 days of development, embryos were maintained in embryo medium containing methylene blue (0.001%) to prevent microbial growth. At 4 days post-fertilization embryos were rinsed in a dilute sodium hypochlorite solution (2 times for 5 min in 0.01% hypochlorite), rinsed with 1% sodium thiosulfate, and then transferred to embryo medium that contained gentamycin sulfate (10 mg/L) to prevent microbial growth. Embryos were not placed in gentamycin sulfate media prior to 4 days post fertilization due to increased mortalities of embryos if exposed before this time. Embryo medium was

changed daily and dead or hatched embryos were removed and dealt with according to proper animal care and safety protocols.

Embryo Staging

Embryos were maintained at 25°C in the dark in an environmental growth chamber (Sheldon Manufacturing, Cornelius, OR) until after they were determined to have entered diapause II. Embryos were determined to have entered diapause II if they were older than 24 days post-fertilization (dpf) with reduced heart rates and a lack of further development such as is observed in escape embryos that bypass diapause II. In order to break diapause, embryos were incubated at 30°C with constant light provided by four 48", full spectrum aquarium light bulbs for 48 hr (Model 2015 Environmental Chamber, Sheldon Manufacturing, Cornelius, OR). Starting at 48 hr, embryos were sorted and those that had broken diapause II were grouped as a synchronous cohort. If necessary, embryos were placed back into the 30°C incubator and checked every 24 hours until the majority had begun to break diapause II.

Embryos were staged according to (Wourms 1972a) using an inverted compound microscope (Leica DMIRB, Wetzlar, Germany). Four developmental time points were sampled across a range of post-diapause II development that represents the full spectrum of anoxia tolerance in this species (Table 1).

Table 1. Developmental stages used in this study. LT₅₀ is defined as the amount of time it takes for an experimental treatment to kill 50% of an experimental population.

Stage	Name	dpd	Description	LT ₅₀ Anoxia, days
32/33	Diapause II	0	dormant embryo, late neurula stage, 38 pairs of somites, heart rate < 20 bpm	41-62
36	Early organogenesis	4	onset of organogenesis, melanocytes present, straight gut tube present, eye pigmentation present, heart rate 60-80 bpm, hemoglobin expressed	65-74
40	Full overgrowth	12	late organogenesis, embryo grown around yolk circumference, gut tube looped, liver present, eye pigmentation dark, heart rate 80-100 bpm	6-7
43	Late prehatching	22	development essentially complete, heart rate 80-100 bpm	<0.5

Exposure to Anoxia

Embryos were exposed to anoxia in an anoxic chamber (Bactron III, Sheldon Laboratories, Cornelius, OR) in which an anaerobic environment was established by purging with a gas mixture of 5%CO₂, 5% H₂, and 90% N₂. The chamber contained a palladium catalyst to ensure removal of any remaining oxygen. Anoxic medium was prepared by purging embryo medium containing 10 mg/l gentamycin sulfate (to prevent microbial growth) with industrial grade compressed nitrogen for 30 min,

and then equilibrating the solution to the atmosphere within the anoxic chamber overnight. The length of time an embryo was exposed to anoxia was dependent on their developmental stage at the beginning of their anoxic exposure, which was about one-half of the LT_{50} for anoxia (Table 1). All groups of embryos were sampled before they were exposed to anoxia to establish an aerobic baseline ($t=0$). Diapause II (DII) embryos were sampled at 2 and 21 days in anoxia, and 1 and 2 d of aerobic recovery from 21 d of anoxia. Stage 36 embryos were sampled at 2 and 34 d in anoxia, and 1 and 2 d of aerobic recovery from 34 d of anoxia. Stage 40 embryos were sampled at 1 and 3 d in anoxia, and 1 and 2 d of aerobic recovery from 3 d of anoxia. Stage 43 embryos were sampled at 0.25 and 0.71 d anoxia, and 1 and 2 d of aerobic recovery from 0.71 d of anoxia.

Embryo Sampling

Anoxic embryos were sampled within the anoxic chamber (Bactron III, Sheldon Manufacturing, Cornelius, OR) by transferring embryos onto a nylon mesh (1 mm mesh size) blotting away excess embryo medium with a dry paper towel, and transferring them into pre-weighed microcentrifuge tubes that were closed before removal from the anoxic chamber. The tubes were then quickly weighed and the embryos flash-frozen by submergence in liquid nitrogen. Aerobic embryos were sampled outside the anoxic chamber in an identical manner. Embryos were stored at -80°C until immediately before use for PEPCK activity assays or extraction of metabolites.

Phosphoenolpyruvate Carboxykinase Enzyme Activity Assays

The capacity for total enzymatic activity of phosphoenolpyruvate carboxykinase (E4.1.1.32, PEPCK) was determined spectrophotometrically (Pharmaspec 1700, Shimadzu, Kyoto, Japan) at 25°C following the method of Lockwood et al. (2012). Embryos were suspended in 4 volumes (by weight) of cold 10mM Tris (pH=7.2 @ 25°C) and homogenized using a ground glass hand homogenizer for 20 turns. The assay buffer consisted of a total volume of 1 ml of 100mM imidazole (pH 6.9 @ 20°C), containing 1mM MnCl₂, 1mM MgCl₂, 20mM NaHCO₃, 1mM GDP, 0.15mM NADH, 5 U ml⁻¹ MDH, and 1mM phosphoenolpyruvate (PEP). Background activity was monitored at 340 nm for 10 min after the addition of 25 µl of 5X embryo homogenate. To initiate the PEPCK reaction, 80 µl of 25 mM PEP was added to the reaction and the decrease in absorbance of NADH at 340 nm was recorded for several minutes. PEPCK activity was calculated as the slope obtained after addition of the PEP after subtracting the background rate with only the embryo homogenate present. Absorbance data were collected using UVProbe software (Ver.2.01, Shimadzu, Kyoto, Japan) and exported to Prism (version 5, GraphPad Software, San Diego, CA) for determination of initial slopes. Slopes were then converted to IU g⁻¹ wet mass or IU g⁻¹ DNA.

Determination of DNA Content

DNA concentration was determined on homogenates previously frozen at -80°C following their initial use for the PEPCK assays (see above). DNA content was determined in order to express PEPCK data in IU g⁻¹ DNA instead of IU g⁻¹ wet mass.

DNA content was measured using the Quant-iT™ dsDNA High-Sensitivity Assay Kit, Broad Range (Molecular Probes, Invitrogen, Grand Island, NY) according to the manufacturer's instructions. An excitation wavelength of 485 nm and emission wavelength of 535nm were used to measure total fluorescence using a microplate reader (Tecan infinite M200 PRO, San Jose, CA) and its companion software (icontrol ver. 1.7). Each standard was run in triplicate and each sample was run in duplicate. A standard curve of fluorescence was created using the standards from the kit for each batch of working reagent.

Preparation of Metabolite Extracts

Groups of 20 embryos were sampled as described above and frozen as whole embryos at -80°C until metabolite extractions were performed. Embryos were homogenized in 1.5 ml of 60% acetonitrile (HPLC grade, Sigma) in a 1.5 ml polypropylene microcentrifuge tube using a polypropylene pellet pestle (USA Scientific). Metabolites were extracted from this homogenate by bringing the final volume to 4.5 ml with final ratios of 2:1.5:1 for acetonitrile:water:chloroform with a final concentration of 1 mM BHT (butylated hydroxytoluene). The phases were mixed thoroughly and then separated by centrifugation at 4,500 x *g* for 20 min at 4°C. The aqueous layer was retained in a 7 ml polypropylene screw-cap vial (USA Scientific). The remaining organic phase and interphase area were transferred to a 1.5 ml polypropylene microcentrifuge tube and 0.5 ml of a 2:1 mixture of chloroform:methanol with a final concentration of 1 mM BHT was added. The phases were mixed and separated by centrifugation at 14,000 x *g* for 20 min at 4°C.

The aqueous layer (top) layer was carefully removed and added to the original aqueous layer obtained above. The extract was then flash frozen in liquid nitrogen and lyophilized overnight. The mass of the dry extract was determined and the powder resuspended in 0.2 ml of water. One eighth of the metabolite extract (0.025 ml) was transferred to a glass vial and lyophilized until dry. This fraction of the extract was then prepared for GC/MS analysis according to the methods outlined in Podrabsky et al. (2007). Briefly, the lyophilized extract was resuspended in 0.05 ml of a 1:1 ratio of acetonitrile: N-methyl-N-[tert-butyldimethylsilyl]trifluoroacetamide (MTBSTFA). The vials were then sealed and sonicated at room temperature for 3 hr.

Gas Chromatography and Mass Spectrometry Analysis of Metabolites

MTBSTFA-derivatized extracts (see above) were analyzed by GC/MS analysis according to methods outlined in Fan et al. (1993). Briefly, 0.5 µl of derivatized extract was injected into a Varian 3400 gas chromatograph (Varian Instruments, Palo Alto, CA) coupled to a Finnigan ITD 806 mass spectrometer (Finnigan, San Jose, CA). A mixture of 30 compounds was treated as above and analyzed in parallel to the samples to serve as calibration standards. GC/MS data were analyzed using Xcalibur software (ver1.3). Values were batch processed and manually analyzed to ensure correct retention times and peak spectra.

Statistical Analysis

Graphical and statistical analyses were performed using Prism software (Version 5.0, GraphPad Software, San Diego, CA). Where appropriate, analysis of variance

was used to compare means both within and between developmental stages and treatments with anoxia. Means were considered statistically different when $p \leq 0.05$.

Results

General Considerations for Metabolite and PEPCK Analysis in Whole Embryos

When analyzing enzyme activity or metabolite data in whole embryos, it is important to note that enzyme activity (or metabolite concentration) is typically expressed per g of wet mass. Data presented in this way represent what is happening in the entire developing embryo. Developing embryos vary in their tissue composition, cell number and DNA content as development progresses, which is not taken into account when biochemical properties are expressed per unit wet mass. Expressing enzymatic activity or metabolite concentrations per g of total DNA is meant to correct for the major differences in cell number that are often associated with progression through development. However, this normalization procedure is based on several assumptions as well, chief of which are that each cell has a roughly equal DNA content and the DNA is contained within the embryonic cells. It is important to keep in mind that concentrations of metabolites or measures of enzyme activity may be higher in the embryonic fish body than the values reported due to the possibility of compartmentalization within the embryo (yolk, embryos, perivitelline fluid) or cell types (neurons, hepatocytes, myocytes).

Phosphoenolpyruvate Carboxykinase Activity

When phosphoenolpyruvate carboxykinase activity is expressed per g wet mass, which represents PEPCK activity of the entire embryo, there was a statistically significant (ANOVA, $p < 0.01$) increase in total PEPCK activity throughout normoxic development from a mean 0.107 ± 0.030 (mean $\pm$ SEM) in DII embryos to $0.402 \pm$

0.091 IU g⁻¹ wet weight in 22 dpd embryos (Fig. 3). All developmental stages exhibited a slight decrease in PEPCK activity as a result of anoxic exposure except for 22 dpd embryos that experienced a modest increase to 0.460 ± 0.089 IU g⁻¹ wet weight. However, no developmental stage exhibited a response that was statistically significant (ANOVA, $p > 0.05$). No significant changes were observed in PEPCK activity during recovery in DII, 4 dpd, or 22 dpd embryos (ANOVA, $p > 0.05$), however 12 dpd embryos had elevated PEPCK activity after 24 hr of aerobic recovery (ANOVA, $p < 0.05$) that returned to control levels by 48 hr of recovery.

When normalized per g of DNA, which represents PEPCK activity per cell, PEPCK activity decreased significantly (ANOVA, $p = 0.024$) during post-diapause II aerobic development, most significantly from 2174 ± 572 in DII embryos to 962 ± 239 IU g⁻¹ DNA in embryos 4 dpd (Fig. 3). Embryos in diapause II and after 4 and 12 days of post-diapause development experienced a decrease in PEPCK activity expressed per g of DNA in response to anoxia (Fig. 3). However, this pattern was only statistically significant for the 4 dpd embryos (ANOVA, $p = 0.03$). Embryos at 22 dpd exhibited no significant changes in PEPCK activity during exposure to anoxia or recovery from anoxia.

Metabolite Analysis

Anaerobic Metabolites

Lactate. Lactate concentration remains relatively low during post-diapause II development under normoxia, but there is a statistically significant (ANOVA, $p < 0.0001$) increase in lactate from 0.443 ± 0.042 mmol kg⁻¹ in diapause II

embryos to concentrations around 1-1.5 mmol kg⁻¹ in embryos between 4 and 22 dpd (Fig. 4). In response to anoxia, lactate concentration increased significantly (ANOVA, $p < 0.0001$) in all developmental stages (Fig. 4). Lactate concentrations remained stable at around 5.097 ± 0.189 mmol kg⁻¹ and 7.901 ± 1.270 mmol kg⁻¹ during aerobic recovery in DII and 4 dpd embryos (respectively), decreased during recovery in 12 dpd embryos, but did not reach control levels, and statistically increased (ANOVA, $p < 0.0001$) during recovery in 22 dpd embryos.

Alanine. Alanine decreased in concentration during aerobic development (ANOVA, $p = 0.0003$) from 1.816 ± 0.274 mmol kg⁻¹ at 4 dpd to 0.566 ± 0.045 mmol kg⁻¹ at 22 dpd (Fig. 4). In response to anoxia, alanine concentrations increased significantly (ANOVA, $p < 0.0003$) in all developmental stages, reaching maximum concentrations of 3.966 ± 0.111 mmol kg⁻¹ in 4dpd embryos after 44 days of anoxia (Fig. 4). Diapause II and 4 dpd embryos showed little change in alanine concentration during aerobic recovery (ANOVA, $p > 0.05$) whereas 12 dpd embryos displayed a significant decrease in concentration (ANOVA, $p < 0.001$) but were not able to fully recover even after 48 hr. Embryos at 22 dpd continued to produce alanine (ANOVA, $p < 0.001$) even during aerobic recovery.

γ -aminobutyrate (GABA). GABA is present in extremely low levels during early post-diapause II development around 0.05-0.2 mmol kg⁻¹, but there is a statistically significant (ANOVA, $p = 0.0012$) increase in GABA in 22 dpd embryos (Fig. 4). Exposure to anoxia results in a significant increase of GABA for all developmental stages (ANOVA, $p < 0.0001$) except for embryos at 22 dpd which did not exhibit a statistically significant increase (ANOVA, $p = 0.158$). Embryos at 4 dpd

showed the highest increase in GABA from 0.034 ± 0.006 at $t=0$ to 107.7 ± 6.296 mmol kg⁻¹ after 44 days of anoxia (Fig. 4). GABA levels did not fall substantially after 48 hr of recovery in any of the developmental stages investigated.

Citric Acid Cycle Intermediates

Fumarate. Fumarate concentrations were low but increased steadily during normoxic development from 0.033 ± 0.002 mmol kg⁻¹ in DII embryos to 0.280 ± 0.010 mmol kg⁻¹ in 22 dpd embryos (Fig. 5). Diapause II and 4 dpd embryos exhibited stable levels of fumarate during prolonged periods of anoxia. In contrast, 12 dpd embryos continued to accumulate fumarate during anoxia while 22 dpd embryos experienced a decline in the concentration of fumarate. During aerobic recovery, DII and 4 dpd embryos maintain stable levels of fumarate, while fumarate decreased in 12 dpd embryos and increased in 22 dpd embryos during aerobic recovery.

c-aconitate. During normoxic development, c-aconitate concentrations show a sharp increase from 0.381 ± 0.047 mmol kg⁻¹ in diapause II to 0.820 ± 0.052 mmol kg⁻¹ in 4 dpd embryos, and then a steady decrease as development progresses toward a low of 0.294 ± 0.011 at 22 dpd (Fig. 5). Diapause II embryos exhibit relatively stable concentrations of c-aconitate during long-term anoxia, while all post-diapause II developmental stages exhibit a decrease in concentration in response to anoxia. Concentrations did not return to pre-anoxic levels for any stage even after 48 hr of aerobic recovery (Fig. 5).

Citrate. Citrate is the most abundant TCA cycle intermediate in embryos of *A. limnaeus* with normoxic concentrations ranging from 8.541 ± 0.469 mmol kg⁻¹ in 4 dpd embryos to 4.168 ± 0.464 mmol kg⁻¹ in 22 dpd embryos (Fig. 5). Citrate levels increase during early post-diapause II development and then decline from 4 – 22 dpd. All developmental stages exhibit a slight decrease in citrate concentration in response to anoxic insult (Fig. 5). During normoxic recovery, citrate concentration increased to levels higher than before exposure to anoxia in DII embryos to a high of 8.001 ± 0.430 mmol kg⁻¹ at 48 hr of aerobic recovery (Fig. 5). Embryos at 12 and 22 dpd recovered to concentrations close to pre-anoxic levels while 4 dpd embryos exhibited no significant change in concentration even after 48hr of aerobic recovery.

Succinate. Throughout normoxic post-diapause development, succinate concentrations remain relatively low but increase significantly (ANOVA, $p < 0.001$) from 0.041 ± 0.004 mmol kg⁻¹ in diapause II embryos to 0.099 ± 0.014 mmol kg⁻¹ in 22 dpd embryos (Fig. 5). In response to anoxic incubation, concentrations of succinate increase across all developmental stages (ANOVA, $p < 0.003$), reaching a high of 1.573 ± 0.145 mmol kg⁻¹ in 4 dpd embryos exposed to 44 days of anoxia (Fig. 5). Succinate concentrations do not change during aerobic recovery in DII embryos. Succinate concentrations decrease in embryos at 4 and 12 dpd and do not return to control levels (ANOVA, $p < 0.05$). In 22 dpd embryos succinate levels increase significantly during the first hour of aerobic recovery (ANOVA, $p < 0.001$).

Malate. Malate levels remain consistently low during post-diapause II development ranging from $0.049 \pm 0.003 \text{ mmol kg}^{-1}$ in 4 dpd embryos to $0.028 \pm 0.003 \text{ mmol kg}^{-1}$ after 22 days of post diapause development (Fig. 5). Diapause II embryos maintain stable low concentrations of malate during prolonged exposure to anoxia, while all three post-diapause II stages accumulate malate in response to anoxia. Embryos at 4 dpd show a modest increase in malate concentrations during anoxia, while 12 dpd embryos exhibit an increase from $0.033 \pm 0.003 \text{ mmol kg}^{-1}$ to $0.393 \pm 0.031 \text{ mmol kg}^{-1}$ after 5 days of anoxia. All developmental stages exhibit an increase in malate during aerobic recovery except for embryos at 12 dpd that instead experience a decrease in about half of the accumulated malate after 48 hr of aerobic recovery (Fig. 5).

α -ketoglutarate. Concentrations of α -ketoglutarate stay extremely low and consistent during normoxic development, ranging from $0.006 \pm 0.001 \text{ mmol kg}^{-1}$ in DII embryos to $0.004 \pm 0.0008 \text{ mmol kg}^{-1}$ after 22 days of post-diapause II development (Fig. 5). Anoxia does not appear to cause major alterations in the concentration of this metabolite in any developmental stages. However, during aerobic recovery all stages experience a small but significant increase in α -ketoglutarate (Fig. 5).

Amino Acids

Essential Amino Acids. All of the essential amino acids in fish that were identified in this study (9 of the 10, missing only tryptophan) appear to exhibit similar developmental changes and similar changes in response to anoxia relatively

independent of developmental stage (Fig. 6). The concentrations of all of these amino acids are lowest in diapause II embryos, increase slightly in 4 dpd embryos, and then tend to decrease during the rest of post-diapause II development. They all tend to increase in concentration in response to exposure to anoxia, and then decrease towards pre-anoxic levels during aerobic recovery (Fig. 6). Worthy of special note are the relatively high levels of lysine in these embryos, and the steep and steady decline in lysine levels during post-diapause II development.

Non-essential Amino Acids. The non-essential amino acids show a variety of patterns associated with normoxic development and exposure to anoxia.

Cysteine. Cysteine levels are relatively low in embryos of *A. limnaeus* but increase from 0.052 ± 0.005 mmol kg⁻¹ in diapause II embryos to a high of 0.243 ± 0.029 mmol kg⁻¹ in embryos at 22 dpd (Fig. 7). All developmental stages respond to anoxia by increasing levels of cysteine, with poor recovery towards pre-anoxic levels even after 48 hr of aerobic incubation (Fig. 7).

Glycine. Glycine exhibits a unique pattern of change throughout normoxic development, decreasing in concentration from 1.005 ± 0.049 mmol kg⁻¹ in DII to a low of 0.566 ± 0.012 mmol kg⁻¹ at 12 dpd and then increasing again to 0.870 ± 0.084 mmol kg⁻¹ by 22 dpd (Fig. 7). When exposed to anoxia, glycine concentrations increase in all developmental stages. While concentrations fall during aerobic recovery in all stages, 48 hr is not sufficient time to reach pre-anoxic levels (Fig. 5).

Hydroxyproline. During normoxic development hydroxyproline concentrations are extremely low, and exhibit an increase from 0.003 ± 0.0002 mmol kg⁻¹ in DII embryos to a peak of 0.037 ± 0.002 mmol kg⁻¹ in embryos at 12 dpd, followed by a slight decrease at 22 dpd (Fig. 7). Concentrations of hydroxyproline do not appear to respond in any major way to anoxia or recovery from anoxia (Fig. 7).

Ornithine. Ornithine concentrations decrease during normoxic development from a high of 0.827 ± 0.072 mmol kg⁻¹ in diapause II embryos to a low of 0.169 ± 0.006 mmol kg⁻¹ in embryos at 22 dpd (Fig. 7). When exposed to anoxia all stages exhibit a decrease in ornithine concentration, with very little return towards pre-anoxic values even after 48 hr of aerobic recovery (Fig. 7).

Proline. Proline concentrations are relatively low in embryos of *A. limneus* with the highest concentrations of 0.243 ± 0.024 mmol kg⁻¹ occurring in 4 dpd embryos (Fig. 7). Proline concentrations increase in all developmental stages in response to anoxia reaching a high of 0.927 ± 0.069 mmol kg⁻¹ in 4 dpd embryos after 44 days of anoxia. Proline concentrations do not return to pre-anoxic levels after 48 hr of aerobic recovery.

Serine. Serine concentrations are similar in DII and 4 dpd embryos with concentrations just under 3 mmol kg⁻¹. However, during normoxic development serine concentrations drop by about 60% in 12 and 22 dpd embryos to around 1 mmol kg⁻¹. When exposed to anoxia, all embryos show an increase in serine concentration, with 22 dpd embryos showing the smallest increase. Aerobic recovery supports a return to near pre-anoxic levels in all stages (Fig. 7).

Tyrosine. Tyrosine concentrations peak at $1.249 \pm 0.132 \text{ mmol kg}^{-1}$ in 4 dpd embryos and then decrease during post-diapause II development to a low of $0.485 \pm 0.030 \text{ mmol kg}^{-1}$ in embryos at 22 dpd (Fig. 7). All developmental stages exhibit an increase in tyrosine concentrations upon exposure to anoxia, with 4 dpd embryos showing the greatest increase to $2.126 \pm 0.205 \text{ mmol kg}^{-1}$ after 44 days of anoxia. Up to 48 hr of aerobic recovery was not sufficient time for concentrations to return to pre-anoxic levels.

TCA Cycle Family Amino Acids. Four amino acids are considered part of this family because of their ability to be rapidly transaminated into TCA cycle keto acids.

Aspartic Acid. Aspartic acid increases in concentration during normoxic development from a low of $0.141 \pm 0.011 \text{ mmol kg}^{-1}$ in DII embryos to a peak of $1.288 \pm 0.105 \text{ mmol kg}^{-1}$ at 12 dpd before declining slightly to $1.103 \pm 0.067 \text{ mmol kg}^{-1}$ at 22 dpd (Fig. 8). In response to anoxia, concentrations of aspartic acid decrease dramatically across all developmental stages. Concentrations of aspartate remained low even after up to 48 hr of aerobic recovery (Fig. 8).

Asparagine. Asparagine concentrations decrease steadily during post-diapause II development from $0.401 \pm 0.042 \text{ mmol kg}^{-1}$ in DII embryos to a low of $0.053 \pm 0.020 \text{ mmol kg}^{-1}$ in embryos at 22 dpd (Fig. 8). When exposed to anoxic conditions, anoxia tolerant embryos (DII and 4 dpd) maintain constant or slightly reduced concentrations of asparagine, while anoxia sensitive stages experience an

increase (Fig. 8). Aerobic recovery from anoxia is associated with a decrease in the concentration of asparagine in all stages investigated.

Other Metabolites

Ascorbate. Ascorbate concentrations remain relatively low and stable around 0.065 mmol kg⁻¹ from diapause II to 12 dpd, and then decreases significantly to 0.028 ± 0.007 by 22 dpd (Fig. 8). Ascorbate levels remain relatively constant after exposure to anoxia and during aerobic recovery from anoxia.

α-glycerol-3-phosphate (G3P). Two distinct peaks are identified via the methodologies used in this study that both represent G3P. Both GC peaks showed similar changes in concentration in post diapause embryos exposed to anoxia, and were added together to represent the total amount of G3P in the embryo extracts. Overall levels of G3P are relatively low in developing embryos, but a slight increase in concentration is observed during post-diapause II development from 0.140 ± 0.023 mmol kg⁻¹ in 4dpd embryos to 0.751 ± 0.115 mmol kg⁻¹ in 22 dpd embryos (Fig. 8). Exposure to anoxia caused a slight increase in the concentration of G3P in all embryonic stages observed, with levels falling to pre-anoxic levels rather quickly upon return to normoxia in all stages except embryos at 22 dpd. 22dpd embryos responded uniquely to aerobic recovery with a slight increase in G3P concentration after 1 hr of recovery.

Discussion

Phosphoenolpyruvate carboxykinase and DNA content

PEPCK activity per gram of embryo increases during post-diapause II development, but PEPCK activity on a per cell basis (indicated by activity expressed per unit DNA) decreases during development. Thus, if PEPCK activity is critical for support of anaerobic metabolism, diapause II embryos are the best prepared for anoxic conditions. In general, PEPCK activity does not appear to change substantially when embryos are exposed to anoxia, although there is a small but statistically significant decrease in 4 dpd embryos. Thus, it appears that metabolic flux through PEPCK during anoxia is likely dependent on the amount of this enzyme present prior to the anoxic exposure. From this perspective, it appears that diapause II embryos have the greatest capacity for PEPCK activity of all the developmental stages, and may be able to support higher fluxes through this pathway. This fact points to a unique physiology of diapause II embryos in terms of metabolic regulation compared to post-diapause II embryos. However, it is unclear from these data if a high amount of PEPCK activity is required to support anoxic metabolism, especially because embryos at 4 dpd have about half of the capacity for PEPCK activity compared to diapause II embryos, but both stages share a similar tolerance to anoxia (Podrabsky et al. 2007, Podrabsky et al. 2012). Additional studies using stable isotope tracers would be required to examine more closely the importance of carbon flux through PEPCK to the support of anaerobic metabolism in this species.

Anaerobic Metabolism and anoxia tolerance in A. limnaeus embryos

Animals able to survive long periods of anoxia tend to depend on fermentation of carbohydrates to sustain their metabolism (Hochachka et al. 2002). Indeed, glycolytic production of lactate appears to be the dominant metabolic end-product in vertebrate tissues forced to rely on anaerobic metabolism (Hochachka et al. 2002), with the notable exception of ethanol production in goldfish and Crucian carp (Shoubridge et al. 1980, Shoubridge et al. 1981, Johansson et al. 1995). Lactate has previously been identified as the major metabolic end product in anoxic *A. limnaeus* embryos with the rate of lactate accumulation highly correlated with survival times in anoxia (Podrabsky et al. 2007). In the present study of diapause II and post-diapause II metabolism during anoxia, lactate is not the major anaerobic end product accumulated, but rather GABA. It is worth noting that the levels of lactate accumulated for diapause II embryos at 32 dpf used in this study are slightly lower (~9 versus ~15 mM) than those reported in previous studies of the same developmental stage when considering time in anoxia (44 d for present study and 60 d for previous studies) and units of expression (mmol/kg embryo, vs mmol/kg water) (Podrabsky et al. 2007). However, this difference is likely attributed to biological variation and perhaps the fact that the previous study used perchloric acid as a metabolite extract where this study used acetonitrile which is less degrading to the metabolites extracted. This study also utilized an anoxic chamber where anoxia was achieved very quickly when compared to the method of the previous study, when embryos were sealed in small vials for extended periods of time.

GABA (γ -aminobutyrate) has been shown to increase in concentration in brain tissue to micromolar levels in several vertebrates that are able to survive extended periods of anoxia where it is thought to act as an inhibitory neurotransmitter to decrease neural activity (Lutz 1992, Lutz et al. 2004, Lutz et al. 2004). GABA is synthesized from glutamate by way of the enzyme glutamate decarboxylase and, in post-synaptic tissue, is degraded to succinate (Turner et al. 1983). *Austrofundulus limnaeus* embryos exhibit a prominent increase in GABA concentrations in response to anoxia at DII, 4dpd, and 12 dpd but not in 22 dpd embryos. In fact, only embryos that can survive anoxia for several days appear to have the ability to produce GABA in large quantities. This has been previously observed in *A. limnaeus* embryos during early development where GABA increased from almost non-existent basal levels during aerobic development to between 7 and 12 mM after 60 days of anoxia, but only in embryos that were able to survive long-term anoxia (Podrabsky lopez 2007).

The levels of GABA accumulated in 4 dpd embryos are nearly an order of magnitude higher than previous reports for early embryos of *A. limneus* and several orders of magnitude greater than in the brain of other anoxia tolerant vertebrates. These levels of GABA are much greater than would be necessary in the context of neuroprotection through inhibitory neurotransmission, and thus it is very likely that GABA plays a different or additional role in supporting anoxia tolerance in embryos of *A. limnaeus*.

The levels of GABA accumulated under anoxia reported here for diapause II embryos at 32 dpf ($\sim 35 \text{ mmol kg}^{-1}$) are considerably higher than previously

reported (15 mM) in (Podrabsky et al. 2007). It is possible that some of the difference is due to biological variation, but it is highly likely that either the exposure methods employed in this study (anoxic chamber versus sealed vials) or the method of extraction (acetonitrile versus perchloric acid) are the cause of the difference. It is most likely the method of anoxic exposure employed in this study, which imposes anoxia much more quickly and completely than sealing embryos into glass vials. Therefore, this study should be considered a more accurate representation of changes in metabolite concentration in response to anoxia.

Citric Acid Cycle Intermediates

Changes in concentrations of TCA cycle intermediates during anoxia imply that the maintenance of mitochondrial metabolism is important to the survival of post-diapause II embryos exposed to anoxia. This is consistent with previous data for pre-diapause embryos of *A. limnaeus* (Podrabsky et al. 2007). A more recent study, (Duerr et al. 2010), found that mitochondria of *A. limnaeus* embryos undergo a reversible suppression of oxidative capacity during diapause and hypothesized that embryos of *A. limnaeus* must maintain a limited citric acid cycle activity during metabolic dormancy. This altered mitochondrial physiology allows for the metabolic transformations associated with the citric acid cycle to proceed, despite low rates of oxidative phosphorylation. This study supports the hypothesis posed in Duerr et al. (2010) by showing that a limited citric acid cycle is maintained during anoxia by post diapause embryos. This is shown through

an increase in concentrations of malate and succinate, but not alpha ketoglutarate after embryos were exposed to anoxia.

TCA Cycle Family Amino Acids

Ammonia is the main nitrogenous waste product produced by most teleost fish. Aspartate has been shown to be the primary source from which ammonia is created by means of a transamination reaction in the teleost liver (Janicki, Lingis 1970). One can imagine that in periods of anoxia, controlling production of ammonia is critical to maintain survival, especially in small hypoxic/anoxic ponds where evaporative water loss can increase concentrations of all substances present. The pronounced decrease in concentration of aspartate across all developmental stages in response to anoxia may decrease the potential for accumulation of waste ammonia to detrimental levels in the environment. Further studies are needed to ascertain the exact role of aspartate and ammonia in anoxia induced metabolic processes.

Conclusions

The results of this study help us to better understand the unique metabolic state of anaerobiosis in post diapause *A. limnaeus* embryos. Post diapause embryos of *A. limnaeus* display shared metabolic traits with other vertebrates such as build-up of lactate and alanine in oxygen limiting conditions, but that is where similarities end. *A. limnaeus* embryos also exhibit a profound ability to produce GABA in oxygen limiting conditions. Increases in malate and succinate were noted in response to

anoxia and are indicative of maintenance of limited citric acid cycle reactions. A decrease in concentration of aspartate was also noted and is hypothesized to play a role in decreasing overall ammonia build up in the system.

However, additional questions remain. For instance, it is not clear by what mechanisms the metabolic changes associated with anoxia tolerance are caused. It is also not clear in which tissues these specific changes take place. Further studies using stable isotope tracers would enable greater resolution when attempting to explain increases or decreases in concentration of metabolites in different tissues in response to anoxia. Understanding how metabolites change and in what tissues could possibly enable discovery of a novel method for survival in extended periods of anoxia.

Figures

Figure 3. Phosphoenolpyruvate carboxykinase activity increases during post-diapause II development when expressed in (a.) IU g^{-1} wet weight and decreases during development when expressed as (b.) IU g^{-1} DNA. Symbols represent means $\pm$ SEM ($n \geq 3$). The data points for aerobic development consist of the $t=0$ sample points for each developmental stage (before anoxic exposure).

Figure 4. Concentrations of anaerobic metabolites present in post diapause embryos of *A. limnaeus* during anoxia and normoxic development. Values are expressed as mm kg⁻¹. Symbols represent means $\pm$ SEM. Hollow symbols and dotted lines indicate normoxic recovery timepoints. The data points for aerobic development consist of the t=0 sample points for each developmental stage (before anoxic exposure).

Figure 5. Concentrations of citric acid cycle intermediates present in post diapause embryos of *A. limnaeus* during anoxia and normoxic development. Values are expressed as mm kg⁻¹. Symbols represent means $\pm$ SEM. Hollow symbols and dotted lines indicate normoxic recovery timepoints. The data points for aerobic development consist of the t=0 sample points for each developmental stage (before anoxic exposure).

Figure 6. Concentrations of essential amino acids present in post diapause embryos of *A. limnaeus* during anoxia and normoxic development. Values are expressed as mm kg⁻¹. Symbols represent means ± SEM. Hollow symbols and dotted lines indicate normoxic recovery timepoints. The data points for aerobic development consist of the t=0 sample points for each developmental stage (before anoxic exposure).

Figure 7. Concentrations of non-essential amino acids present in post diapause embryos of *A. limnaeus* during anoxia and normoxic development. Values are expressed as mm kg⁻¹. Symbols represent means $\pm$ SEM. Hollow symbols and dotted lines indicate normoxic recovery timepoints. The data points for aerobic development consist of the t=0 sample points for each developmental stage (before anoxic exposure).

Figure 8. Concentrations of aspartate, asparagine, ascorbate, AG3P, and PO_4 present in post diapause embryos of *A. limnaeus* during anoxia and normoxic development. Values are expressed as mmol kg^{-1} . Symbols represent means $\pm$ SEM. Hollow symbols and dotted lines indicate normoxic recovery timepoints. The data points for aerobic development consist of the t=0 sample points for each developmental stage (before anoxic exposure).

a. Aerobic Conditions

b. Anoxic Conditions

Figure 9. Metabolic pathway of PEPCK during a. aerobic conditions and b. during anoxic conditions. In the absence of oxygen, pyruvate kinase activity is limited and PEPCK creates OAA rather than PEP, which feeds the citric acid cycle.

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