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Carbohydrate and amino acid metabolism in fasting and aestivating African lungfish (*Protopterus dolloi*)

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ABSTRACT

The potential importance of carbohydrates and amino acids as fuels during periods of fasting and aestivation in the African lungfish, *Protopterus dolloi*, were examined. No significant decreases in tissue glycogen levels were observed following 60 days of fasting or aestivation, suggesting lungfish may undergo 'glycogen sparing'. Yet glycogenolysis may be important during aestivation based on the differing responses of two flux-generating enzymes of the glycolytic pathway, hexokinase (HK) and pyruvate kinase (PK). PK is required for glycogen breakdown whereas HK is not. HK activity is significantly down-regulated in the heart and gill tissues during aestivation, while PK activity is sustained. The significant negative correlation between the activity of HK and glucose levels in the heart of aestivating lungfish suggests HK may be regulated by glucose concentrations. There was no indication of anaerobic glycolytic flux during aestivation as lactate did not accumulate in any of the tissues examined, and no significant induction of lactate dehydrogenase (LDH) activity was observed. The increase in glutamate dehydrogenase (GDH) and aspartate aminotransferase (Asp-AT) activities in the liver of aestivating *P. dolloi* suggests some energy may be obtained via increased amino acid catabolism, leading to the generation of tricarboxylic acid (TCA) cycle intermediates. These findings indicate the importance of both carbohydrate and amino acid fuel stores during aestivation in a phylogenetically ancient, air-breathing fish.

1. Introduction

Many animals undergo seasonal metabolic depression to survive environmental stresses (e.g. cold or desiccation). They inhabit sheltered sites such as underground burrows and often experience a 60–99% reduction in their metabolic rate (Guppy and Withers, 1999). Depressing an organism's metabolism involves many physiological and biochemical changes and much work has been dedicated to understanding these adjustments (see reviews by Storey and Storey, 1990; Guppy et al., 1994; Guppy and Withers, 1999; Storey, 2002). Metabolic depression allows an organism to survive for extended periods of time on internal fuel reserves as ATP demand is reduced. During these periods of dormancy, energy may be produced from the catabolism of lipids, ketone bodies (see Frick et al., 2008), carbohydrates or proteins/amino acids and the primary fuel preference can vary between species. In most vertebrates, lipids make up the primary fuel source, followed by protein (see review by Storey, 2002). Carbohydrates typically contribute a relatively small proportion of the total energy budget of vertebrates. For example, spadefoot toads (*Scaphiopus couchii* and *S. multiplicatus*) obtain 72% of energy from fatty acid oxidation, 23% from protein, and only 5% from carbohydrate (Jones, 1980). Yet many vertebrate species accumulate large glycogen stores prior to entering into a dormant period, slowly metabolizing this fuel throughout aestivation (Donohoe and Boutilier, 1998; Jackson, 2000; Jackson et al., 2001; Moraes et al., 2005) suggesting the metabolism of carbohydrates must be maintained.

African lungfish often inhabit shallow ephemeral waters, and during the dry season (typically 4–6 months (Janssens, 1964)), retreat into a mud burrow, secrete a cocoon and enter a state of aestivation. Although the metabolic rate of African lungfish (i.e. *Protopterus aethiopicus*) has been found to decrease by more than 50% during aestivation (Smith, 1930; Delaney et al., 1974; Fishman et al., 1986) sufficient fuel stores must be available to ensure survival. In a separate study (Frick et al., 2008), the importance of lipids and ketone bodies as energy fuels in *P. dolloi* were examined, under control, fasting and aestivating conditions. This paper will address the potential contributions of carbohydrates and amino acids to the energy demands of *P. dolloi*. Carbohydrate metabolism may be important in lungfish during periods of food deprivation, as these fish have large stores of

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glycogen in their heart, liver and red and white muscle, and can use this energy substrate both aerobically and anaerobically (Hochachka and Hulbert, 1978).

During aestivation, African lungfish (*P. aethiopicus*) experience a decrease in heart rate (from ~25 to 15 bpm) (Delaney et al., 1974; Fishman et al., 1986). When an animal's heart rate slows, the delivery of oxygen to the peripheral tissues is reduced, potentially resulting in functional hypoxia or anoxia (Belkin, 1968). Aestivating *P. aethiopicus* experience a decrease in arterial PO_2 (PaO_2) (from ~50 to 25 mmHg) (Delaney et al., 1974; Fishman et al., 1986) and the down-regulation of a key enzyme in the TCA cycle (citrate synthase (CS)) and the electron transport chain (ETC) (cytochrome c oxidase (CCO)) (Frick et al., 2008). In other fish species, reductions in PaO_2 to 20 and 26 mmHg have resulted in lactate accumulation suggesting anaerobic glycolysis is activated (Burggren and Cameron 1980; Boutilier et al., 1988). This led us to explore the possibility that African lungfish display an increased reliance on anaerobic metabolism during aestivation.

It is well known that African lungfish accumulate urea within their tissues during aestivation with the nitrogen coming from amino acids (e.g. Smith, 1930; Janssens, 1964; Chew et al., 2003a; 2004), however; the extent to which amino acids are used for energy production is not clearly understood. Analysis of the activities of enzymes involved in amino acid metabolism will help elucidate the importance of particular pathways of amino acid metabolism and the potential changes taking place during fasting and aestivation. Although amino acid catabolism continues during aestivation, as evident by the accumulation of urea, it may be down-regulated.

Our knowledge of the basic metabolic organization of carbohydrate and amino acid metabolism in this phylogenetically ancient air-breathing fish under normal conditions and under the influence of food deprivation and aestivation is limited. We hypothesized that *P. dolloi* utilizes glycogen stores during both fasting and aestivation, and that glycolytic flux contributes to the energy demand during aestivation. Additionally, during aestivation key enzymes of amino acid metabolism are down-regulated to minimize the production of toxic nitrogenous waste products. To address these hypotheses, the following parameters were measured in control, fasted and aestivated lungfish: 1) the activities of key enzymes involved in the pathways of amino acid and carbohydrate metabolism, 2) the levels of lactate, glucose and glycogen within selected tissues and 3) in order to consider the potential influence of dehydration effects on these parameters, plasma osmolality, percent water content of white muscle tissue and tissue protein concentrations were examined.

2. Materials and methods

2.1. Animals and experimental setup

African lungfish (*P. dolloi*) were purchased from a local fish supplier in Singapore and shipped to the University of Guelph Hagan Aqualab (Guelph, ON, Canada). Fish were kept in freshwater at 25 °C under a 12:12 L:D artificial photoperiod and fed a mixed diet of frozen blood worms (Chironomidae larva) and trout chow pellets (Martin Feeds, Elmira, Ontario) for ~12 months before the commencement of the experiment.

P. dolloi (58.8±4.7 g) were divided into three treatment groups (N=8 for each) 1) control (held in water and fed every second day) 2) aestivated and 3) fasted (held in water but without food for the duration of the experiment (60 days)). There were no significant differences in fish mass between experimental groups (P=0.36). Aestivation was induced using the method described by Delaney et al. (1974) where fish were placed in a muslin sack in the position in which they naturally aestivate in a mud burrow (vertically with their head upwards wrapped under their tail). The sack was suspended in a container filled with water and the water level was lowered ~1 cm/day until the sac was completely dry (5–7 days). In a preliminary study a lungfish was returned to water following 3 weeks aestivation and was observed to recover fully. As these lungfish live in the complete absence of water and form a complete cocoon (with a breathing hole), we consider them to be fully aestivated.

2.2. Tissue and plasma collection

Blood samples were taken from non-anaesthetized fish by caudal puncture using heparinized syringes (500 U mL^{-1}) with a 22-gauge needle. Blood samples were centrifuged at 5000 ×*g*, at 4 °C for 5 min, and plasma was quickly frozen and stored at − 80 °C. Fish were killed by a blow to the head and tissues (liver, gill, heart, kidney, white muscle, and lung) were rapidly excised and immediately frozen in liquid nitrogen and stored at − 80 °C for future analysis, except for a portion of white muscle which was used to determine the percent water content of the muscle. Note: periphery musculature in lungfish is a mosaic of red and white muscle while the remaining is comprised mostly of white muscle (Dunn et al., 1981). In the current study we attempted to only sample white muscle.

2.3. Determination of plasma osmolality and glucose concentrations

Plasma osmolality (mmol kg^{-1}) was determined using a vapour pressure osmometer (Model 5500, Wescor, Utah, USA). Plasma glucose concentration (mmol l^{-1}) was measured using a Sigma diagnostics kit (510-A) (Sigma Diagnostics, St. Louis, MO, USA), which used glucose oxidase for an enzymatic determination of glucose levels. Values are expressed as the mean values of duplicate measurements of each plasma sample.

2.4. Determination of muscle water content

White muscle tissue samples were weighed (~3 g), placed into an aluminum dish and dried at 80 °C. Samples were weighed regularly until a stable dry weight was obtained, at which point the percent water content and the mL of water per g dry tissue were calculated.

2.5. Determination of protein concentrations

The water-soluble protein concentration of the supernatants used to measure enzyme activities were determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.), standardized with bovine serum albumin (BSA).

2.6. Tissues metabolites

Tissues (liver, white muscle, heart and kidney) were ground to a fine powder under liquid nitrogen using a mortar and pestle and added to 10% perchloric acid. An aliquot of this homogenate was used in the determination of glycogen. The homogenate was then centrifuged for 10 min at 7650 ×*g*. The resulting supernatant was neutralized using solid $KHCO_3$ and used in the determination of tissue lactate and glucose levels using a Cary 50 Bio spectrophotometer (Varian Inc., Palo Alto, CA). The method of Gutmann and Wahlefeld (1974) was used to measure tissue lactate levels while glycogen and glucose were determined using the method of Keppler and Decker (1974), where glycogen is hydrolysed and the amount of free glucose in the original sample is subtracted. Lactate and glucose values are presented in µmol g wet $tissue^{-1}$ while glycogen levels are in µmol of glucosyl units g wet $tissue^{-1}$ (N=7 for each).

2.7. Enzyme analysis

2.7.1. Tissue preparation

Tissues (liver, gill, heart, kidney, white muscle, and lung) were thawed and homogenized in ice cold 50 mmol l^{-1} imidazole buffer (pH