METABOLIC ADJUSTMENTS DURING SEMI-AESTIVATION OF THE MARBLE SWAMP EEL (*Synbranchus marmoratus*, Bloch 1795) – A FACULTATIVE AIR BREATHING FISH

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ABSTRACT

Metabolic changes, principally in intermediary metabolism and nitrogen excretion, were investigated in the marble swamp eel (*Synbranchus marmoratus*) after 15 and 45 days of artificially induced semi-aestivation. Glucose, glycogen, lactate, pyruvate, free amino acids, triglycerides, ammonia, urea, and urate contents were determined in liver, kidney, white muscle, heart, brain, and plasma. Lactate dehydrogenase, glutamate dehydrogenase, malate dehydrogenase, aspartate amino transferase, alanine amino transferase, glutamine synthase, ornithine carbamoyl transferase, and arginase enzymes were assayed. The teleost *S. marmoratus* maintained initial energetic demands by lipid oxidation. The course of normal oxidative processes was observed through tissue enzyme profiles. After the lipid stores were exhausted, the fish consumed body proteins. Constant values of hematocrit during induced semi-aestivation suggested that the water balance remained normal. Therefore, the surrounding water was probably did not trigger the semi-aestivation in this teleost. Decrease of ammonia and increase of renal urea synthesis after 45 days of semi-aestivation led to the assumption that an alternative form of eliminating ammonia exists. Metabolic changes entailed by starvation were proposed to explain the biosynthesis of small molecules involved in the semi-aestivation of *S. marmoratus*.

Key words: semi-aestivation, metabolism, adaptation, *Synbranchus marmoratus*, nitrogen excretion.

RESUMO

Ajustes metabólicos do muçum (*Synbranchus marmoratus,* Bloch 1795) – um peixe de respiração aérea facultativa – durante semi-estivação

Foram investigadas variações metabólicas em muçum (*Synbranchus marmoratus*) após 15 e 45 dias de semi-estivação artificial induzida, com ênfase no metabolismo intermediário e excreção de nitrogênio. Glicose, glicogênio, lactato, piruvato, aminoácidos livres, triglicerídios, amônia, uréia e uratos foram determinados em fígado, rim, músculo branco, coração, cérebro, e plasma. As enzimas lactato desidrogenase, glutamato desidrogenase, malato desidrogenase, aspartato amino transferase, alanina amino transferase, glutamina sintetase, ornitina carbamil transferase e arginase foram ensaiadas. O teleósteo *S. marmoratus* manteve sua demanda energética inicial pela oxidação de lipídios. A seqüência normal dos processos oxidativos foi observada por meio do perfil enzimático. Após o consumo das reservas lipídicas, os peixes passaram a consumir proteína. Valores constantes de hematócrito durante a semi-estivação induzida sugeriu que o balanço de água foi mantido normal. Todavia, a água ambiental provavelmente não foi o sinal inicial da semi-estivação em *S. marmoratus*. A diminuição de amônia e o aumento da síntese de uréia renal após 45 dias de semi-estivação levaram a assumir que existe uma forma alternativa de eliminar amônia. As variações metabólicas conseqüentes do jejum prolongado foram propostas como responsáveis pela síntese de pequenas moléculas envolvidas na semi-estivação de *S. marmoratus*.

Palavras-chave: semi-estivação, metabolismo, adaptação, *Synbranchus marmoratus*, excreção de nitrogênio.

INTRODUCTION

Freshwater Neotropical organisms are often exposed to drastic environmental fluctuations. Among these, the most significant are oxygen concentrations (Graham *et al.*, 1978; Dunn *et al.*, 1983), tides (Brooks & Storey, 1997), temperature (Brooks & Storey, 1997), and even water availability in several temporary lagoons and ponds (Val, 1995). External changes trigger immediate organismal responses to cope with stressors (Romer, 1966; Thomson, 1980; Bray, 1985; Long, 1995). Among the various organic strategies, metabolic arrest is a mechanism observed in many amphibians, reptiles, and mollusks (Abe, 1995; Brooks & Storey, 1997). This response, usually called aestivation, seems to be initiated by absence of oxygen (Brooks & Storey, 1997; Hochachka *et al.*, 1997); however, this is not common in fish. Nevertheless, during dry periods, a number of species exposed to air have been reported to aestivate in mud (Rosen & Greenwood, 1976; Bicudo & Johansen, 1979). Although these fish are air-breathing species, impairment of oxygen uptake may occur, which would justify aestivation. However, lack of surrounding water has also been proposed as a probable signal for observed metabolic arrest (Hochachka & Guppy, 1987). Reduced oxygen availability leads to several metabolic changes, e.g., lactate increase and glycogen mobilization (Hochachka & Somero, 1984), and even a need for protein catabolism (Dave *et al.*, 1975; Mommsen *et al.*, 1980; Smith, 1981; Wood & Fung, 1981; Moon, 1983). Moreover, the absence of surrounding water requires nitrogen metabolism adjustments.

The impossibility of eliminating ammonia under such circumstances results in hyperammonemia, which requires alternative ways to avoid toxicity (Walsh *et al.*, 1990; Barber & Walsh, 1993; Walsh *et al.*, 1994). Synthesis of glutamine has been reported in air-breathing fish (Iwata & Kakuta, 1983) and glutamine accumulation has been observed in the brain of various teleosts exposed to high levels of ammonia (Korsgaard *et al.*, 1995). The facultative air-breathing fish *Oxyeleotris marmoratus* activates hepatic glutamine synthase during exposure to air (Jow *et al.*, 1999). However, ammonia can be also be wasted through urea synthesis. A set of ornithine-urea cycle enzymes has been reported for some teleost species. Moreover, specific environmental conditions such as those created by ammonia increase (Saha & Ratha, 1994; Saha *et al.*, 1999; Moraes & Polez, 2004b), alkaline waters

(Randall *et al.*, 1989; Moraes & Polez, 2004a), and confinement and/or crowding (Walsh *et al.*, 1990; Walsh & Milligan, 1995) augment the presence of these enzymes. The Neotropical facultative air-breather *Synbranchus marmoratus* (marble swamp eel) is able to survive up to three months embedded in mud (Bicudo & Johansen, 1979), carrying out the gas-exchange process in the buccopharyngeal epithelium and in the branchial chamber. Under these inhospitable conditions, the animal, in a typically torpid state, sometimes stretches the body, sliding on its own mucus within the mud tunnels. This behavior, called semi-aestivation, requires adjustments in the overall metabolism, thereby raising the following questions: Is this species highly tolerant to ammonia or does the presence of ammonia increase trigger a strategy to overcome toxicosis? Does the absence of surrounding water, by impairing oxygen exchange, result in cellular fermentation? Is the reduced availability of water a signal likely to cause semi-aestivation? The aim of this study was to correlate the metabolic responses of the marble swamp eel with the induced semi-aestivation process.

MATERIAL AND METHODS

Adult *S. marmoratus* weighing 300g ± 50 were collected in late spring from the Tietê River, Ibitinga, São Paulo, Brazil, (48º49'W 21º45'S) and taken to the laboratory. Their new environment consisted of 250 L tanks with clean, filtered water at 25ºC, constant aeration, and natural photoperiod. The fish were acclimatized for 30 days and fed on live bait. After acclimation, 24 fish were transferred to a wide fiberglass box containing 15 cm of mud covered with 15 cm of water. The water was then slowly drained, leaving the box completely empty after 48 hours. When the fish detected the drainage they burrowed into the mud. Two periods of semi-aestivation (15 and 45 days) were assayed. Another group of 24 fish were taken from the clean water tanks, transferred to identical conditions described above, but kept without drainage (control). After 24 h, eight fish were sampled (time-control zero). The sixteen remaining fish were sampled at the end of the first span (day 15) and at the end of the trials (day 45). The fish were randomly collected after day 15 and day 45, transferred to glass aquaria, and anesthetized with 0.27 g/L of MS222, following which blood samples were taken. The animals were then killed, and liver, brain, kidney, heart, and white muscle excised and immediately frozen into liquid nitrogen.

Plasma extract

Whole blood aliquots were used in hematocrit and pH determinations. Blood samples were centrifuged at 12,000 x G for 3 min. To each proper aliquot of supernatants was added 20% trichloroacetic acid (TCA), followed by centrifugation at 12,000 x G for 3 min. The supernatants were used as acid extracts.

Tissue extracts

Liver, brain, kidney, heart, and white muscle were mechanically homogenized 1:10 with 20% TCA using 30 s strokes of a 2,000 rpm motor-driven Teflon pestle. The acid homogenates were centrifuged at 12,000 x G for 3 min, and glucose and lactate were determined in the supernatants. Amino acids were quantified in homogenized liver, kidney, and white muscle, as described above, in 5 volumes of cold distilled water. The homogenates were centrifuged at 12,000 x G for 3 min and 1 volume of 0.3N Ba $(OH)_2$ and 5% $ZnSO_4$ was added to the supernatant. The cloudy, white precipitate was centrifuged at 12,000 x G for 3 min and the pellets discarded.

Metabolites

Metabolic intermediates were colorimetrically determined. Reducing sugars were estimated with phenol-sulphuric acid (Dubois *et al.*, 1956); lactate, with p-hydroxiphenyl phenol (Harrower & Brown, 1972); pyruvate, by dinitrophenyl hydrazine-NaOH (Lu, 1939); free amino acids, by ninhidrine (Copley, 1941); ammonia, by nesslerization (Gentzkow & Masen, 1942); urea, by diacetyl monoxime (Rahmatullah & Boyde, 1980); and urate, by carbonate-phosphotungstate (Henry *et al.*, 1957). Triglycerides were determined by the enzymatic-colorimetric test (McGowan *et al.*, 1983).

Tissue samples from the liver, kidney, and white muscle were completely dissolved in 10 volumes of 6N KOH in a boiling-water bath for 3 min to determine glycogen (Bidinotto *et al.*, 1997). The extract was transferred 1:10 v/v to ethanol; 1 volume of 10% K_2SO_4 was added to improve precipitation. The pellet was re-suspended in 3.0 ml of distilled water and glucose was determined in a proper aliquot using phenol-sulfuric acid (Dubois *et al.*, 1956).

Enzyme assay

Liver, kidney, brain, heart, and white muscle were mechanically homogenized with four 15 sec strokes in a cold-water bath with a motor-driven Teflon pestle at 2,000 rpm. Homogenization was carried out in 0.1M sodium phosphate buffer, pH 7.0, centrifuged at 5,000 x G for 5 min, and the supernatant was used as an enzyme source to determine alanine amino transferase (ALAT) (EC 2.6.1.2) and aspartate amino transferase (AAT) (EC 2.6.1.1) (Reitman & Frankel, 1957); lactate dehydrogenase (LDH) (EC 1.1.1.27), malate dehydrogenase (MDH) (EC 1.1.1.37), and glutamate dehydrogenase (GDH) (EC 1.4.1.2) (Hochachka *et al.,* 1978); glutamine synthase (GS) (EC 6.3.1.2) (Vorhaben *et al.*, 1973), ornithine carbamoyl transferase (OCT) (EC 2.1.3.3) (Nakamura & Jones, 1970), and arginase (ARG) (EC 3.5.3.1) (Rahmatullah & Boyde, 1980). All enzymes were assayed at 25°C.

Statistics

The data are presented as averages ± SD. The significance of differences between averages ($p < 0.05$) was tested using two-way variance analysis (ANOVA). Whenever differences between averages were found, Tukey-Kramer multiple comparison tests were applied.

Results

Significant metabolic adjustments were observed in the semi-aestivated marble swamp eel. Hematocrit (45%) and blood pH (7.7) remained unaltered during both aestivation spans. The liver, kidney, and white muscle glycogen bulk decreased 56%, 46%, and 36%, respectively. Glucose dropped in liver, remained constant in kidney and heart, and rose in brain and white muscle. Pyruvate concentration increased in kidney and white muscle. Lactate concentration was slightly reduced in the liver and markedly so in other tissues. A sharp increase of amino acids was observed in liver, kidney, and white muscle.

These data are presented in Table 1. Plasma pyruvate and amino acids increased after 45 days of semi-aestivation. Plasma triglyceride concentration decreased to undetectable values, while glucose declined significantly. Plasmatic ammonia fluctuated throughout the semi-aestivation period, increasing after 15 days and slowing down at the end. Plasma urea increased and urate did so only after 15 days of semi-aestivation, reaching close to basal levels after 45 days. These results are located in Table 2.

Changes were observed in the activity of the intermediary metabolism enzymes AAT, ALAT, GDH, LDH, and MDH, as shown in Table 3. The aminotransferases AAT and ALAT increased in liver and white muscle, particularly after 15 days of semi-aestivation. Glutamate dehydrogenase increased slightly in white muscle, but showed a significant increase in the liver, heart, and brain. Lactate

dehydrogenase remained constant in the liver but increased in the heart and brain, while malate dehydrogenase increased only in the heart and brain. The enzymes GS, OCT, and ARG involved in nitrogen excretion were assayed in liver and kidney tissue.

Except for arginase, the liver and kidney showed different responses (Table 4). Glutamine synthase and ornithine carbamoyl transferase decreased in the liver and increased in the kidney. Arginase increased significantly in both liver and kidney.

TABLE 1

Metabolic profile of *S. marmoratus* under induced semi-aestivation.

Tissue	Δt	Metabolite				
		Glycogen	Glucose	Pyruvate	Lactate	FAA
Liver	C	308 ± 63	275 ± 31	0.63 ± 0.03	20.42 ± 10.1	1.5 ± 0.1
	A_{15}	365 ± 35	280 ± 21	0.83 ± 0.03	22.23 ± 6.8	1.6 ± 0.1
	A_{45}	$135 \pm 20*$	$190 \pm 16*$	0.97 ± 0.09	15.74 ± 5.6	$19.7 \pm 0.1*$
Kidney	C	62 ± 3	25 ± 1	0.27 ± 0.02	18.77 ± 3.5	2.1 ± 0.2
	A_{15}	$32 \pm 5*$	21 ± 3	0.47 ± 0.03	$11.08 \pm 3.8*$	1.4 ± 0.1
	A_{45}	$33 \pm 4*$	24 ± 4	$1.18 \pm 0.16*$	$8.12 \pm 1.5*$	$16.3 \pm 3.5*$
White muscle	C	11 ± 1	20 ± 2	0.55 ± 0.05	22.41 ± 2.3	4.3 ± 0.9
	A_{15}	9 ± 1	23 ± 4	$0.76 \pm 0.08*$	$25.15 \pm 1.3*$	$2.0 \pm 0.2*$
	A_{45}	$7 \pm 1*$	$28 \pm 2*$	0.46 ± 0.04	20.81 ± 2.3	$15.3 \pm 1.7*$
Heart	C	n-m	88 ± 9	n-m	28.44 ± 8.5	n-m
	A_{15}	n-m	67 ± 7	n-m	22.85 ± 2.5	n-m
	A_{45}	n-m	95 ± 9	n-m	$18.01 \pm 2.7*$	n-m
Brain	C	n-m	19 ± 3	n-m	30.43 ± 5.6	n-m
	A_{15}	n-m	19 ± 2	n-m	28.55 ± 2.65	n-m
	A_{45}	n-m	$25 \pm 2*$	n-m	$15.16 \pm 2.69*$	n-m

Glycogen is expressed as µmol of glucosyl-glucose/g of wet tissue; the other metabolites are expressed as µmol/mg of wet tissue. FAA = free amino acids, C = control, A_1 = aestivation for 15 days, A_2 = aestivation for 45 days, (*) significantly different compared to the control ($p < 0.05$). n-m = not measured.

TABLE 2

Plasma metabolite of *S. marmoratus* under induced aestivation.

Metabolite	Condition		
	C	A_{15}	A_{45}
Glucose	7.90 ± 0.85	$5.38 \pm 0.83*$	$4.07 \pm 0.63*$
Pyruvate	0.19 ± 0.03	0.23 ± 0.02	$0.29 \pm 0.01*$
Lactate	2.16 ± 0.09	2.43 ± 0.26	2.01 ± 0.54
FAA	4.25 ± 0.99	3.94 ± 0.36	$9.55 \pm 0.80*$
TG	52.71 ± 5.42	$11.85 \pm 0.68*$	n-d
Ammonia	3.02 ± 0.05	$3.98 \pm 0.32*$	$0.80 \pm 0.09*$
Urea	0.89 ± 0.19	1.27 ± 0.18	$1.93 \pm 0.11*$
Urate	0.21 ± 0.09	$0.49 \pm 0.05*$	0.22 ± 0.02

Triglycerides are expressed in mg/dL of plasma; the other metabolites are expressed as µmol/mg of wet tissue. FAA = free amino acids, TG = triglycerides, C = control, A_1 = aestivation for 15 days, A_2 = aestivation for 45 days, n-d = not detected, (*) significantly different compared to the control ($p < 0.05$).

TABLE 3

Enzyme response of *S. marmoratus* to induced aestivation.

Tissue	t	Enzyme									
		AAT		ALAT		GDH		LDH		MDH	
Liver	C	2.64	0.21	3.85	0.35	12.44	1.39	3.4	0.3	18.8	2.1
	A_{15}	8.98	0.93*	9.80	0.91*	13.84	1.31	3.3	0.3	18.4	2.1
	A_{45}	13.30	0.96*	4.05	0.20	22.30	2.90*	3.2	0.3	17.9	1.2
White muscle	C	1.74	0.28	1.47	0.18	9.12	1.32	414.1	24	1.4	0.1
	A_{15}	3.02	0.30*	2.58	0.27*	6.93	0.82	240.3	28*	1.3	0.1
	A_{45}	0.72	0.01	1.71	0.13	11.53	1.15	394.1	6	1.4	0.1
Heart	C	n-a		n-a		5.09	0.86	115.4	10	23.4	1
	A_{15}	n-a		n-a		9.89	1.23*	232.1	14*	47.3	3*
	A_{45}	n-a		n-a		8.50	0.68*	267.2	18*	43.4	2*
Brain	C	n-a		n-a		4.18	0.63	81.2	8	6.1	0.6
	A_{15}	n-a		n-a		5.89	0.35*	118.3	11*	9.9	0.9*
	A_{45}	n-a		n-a		5.92	0.68*	104.4	11	8.3	0.5*

AAT = aspartate amino transferase, ALAT = alanine amino transferase, GDH = glutamic dehydrogenase, LDH = lactic dehydrogenase, MDH = malic dehydrogenase, C = control, A1 = aestivation for 15 days, A2 = aestivation for 45 days, (n-a) not assayed, (*) significantly different compared to the control ($p < 0.05$).

TABLE 4

Nitrogen metabolism enzymes of *S. marmoratus* under induced semi-aestivation.

Tissue	t	Enzyme		
		GS	OCT	ARG
Liver	C	0.10 0.01	0.42 0.02	13.1 1.0
	A_{15}	n-d	0.32 0.02*	17.1 1.9
	A_{45}	n-d	0.03 0.00*	21.5 2.1*
Kidney	C	0.05 0.00	0.07 0.01	10.9 0.9
	A_{15}	0.10 0.01*	0.35 0.03*	5.2 0.5*
	A_{45}	0.12 0.01*	0.36 0.04*	17.9 1.6*

GS = glutamine synthase, OCT = ornithine carbamoyl transferase, ARG = arginase, C = control, A1 = aestivation for 15 days, A2 = aestivation for 45 days, (n-d) not detected, (*) significantly different compared to the control ($p < 0.05$).

DISCUSSION

A number of aquatic organisms, particularly freshwater ones, are occasionally exposed to harsh environmental conditions. Droughts, for instance, may reduce lakes and lagoons to small ponds containing predominantly mud and very little water, consequently threatening the lives of many species, particularly fish. For them to survive to continue in such conditions requires special adaptive mechanisms for coping with many stressors, such as temperature, decrease of oxygen, water turbidity, and even predators. Some teleost species adopt similar behaviors to withstand such circumstances. The lungfish *Protopterus* and *Lepidosiren*, for example, dig into the mud to aestivate (Hochachka & Somero, 1971). In drought conditions, the bi-modal breathing fish *S. marmoratus* lies in little tunnels and covers itself with mucus, so remaining until the rainy season begins (Bicudo & Johansen, 1979).

Metabolic demands of the fish must be supplied by organismal storages during the dormant state.

An outcome of induced semi-aestivation in the marble swamp eel was the increase of free amino acids in all assayed tissues, which suggested body-protein consumption. Supplying metabolic demands in this way has previously been reported in eels (Dave *et al.*, 1975; Moon, 1983). However, lipid breakdown would be more advantageous from many points of view. Such a metabolic option has just been reported in the sea bass *Dicentrarchus labrax* when starved; however, a very stressing condition does not necessarily imply aestivation (Echeverría *et al.*, 1997). The exhaustion of fat stores in the marble swamp eel was inferred from the complete depletion of plasma triglycerides after 45 days of semi-aestivation. Lipid catabolism is a metabolic choice providing high energy yield, but requiring fully functioning oxidative pathways.

The oxidative process during semi-aestivation can be assumed from the tissue enzyme profile. Both glycolytic and oxidative metabolisms have been previously reported in the very specialized cardiac muscle of *S. marmoratus* (French & Hochachka, 1978). Indeed, the observed increase of heart dehydrogenases activity is probably the way this species makes up for reduced primary metabolite production. Lactate produced by other tissues has been proposed as an energy source for heart muscle (Collins & Anderson, 1997; Hochachka & Hulbert, 1978). The fermentative picture observed in white muscle of the marble swamp eel bears out this proposition. The increase of LDH and white muscle lactate suggests a fermentative preference at the beginning of the process. However, at the end of a long-term semi-aestivation the metabolic responses of both white and cardiac muscles converged to a similar pattern of physiological adjustment.

The oxidative profile of the hepatic tissue remained unchanged throughout the semi-aestivation period. However, increased activity of nitrogenous metabolism was evidenced by transaminases plus the free amino-acids outline. The lessening of hepatic glycogen and glucose suggest the role of the liver as a glucose supplier through glycogenolysis and gluconeogenesis.

The nervous system of marble swamp eel may have been the main consumer of glucose released by liver. Brain catabolism remained constant throughout the semi-aestivation span. The observed increase of dehydrogenases activity was likely triggered to maintain cell viability; lactate was probably used as energy supplier. Therefore, the general metabolic preference of the brain in *S. marmoratus* under semi-aestivation was basically aerobic, and the oxygen approach was independent of surrounding water.

Considering that hematocrit was kept constant during the trials, the water balance is supposed not to have been impaired; hence, it is difficult to assume any role of the external water supply as a signal to begin semi-aestivation. The observed increase of plasma amino acids should be the result of high protein catabolism. The rise in plasma ammonia concentration might have been expected, however, it decreased about fourfold while the urea doubled. These values are very close to an equimolar rate and, therefore, very suggestive of urea synthesis as a strategy for eliminating ammonia in the absence of environmental water. The kidney seems pivotal in such a process, and the arginase increase was likely the primary consequence of amino acid catabolism. This hypothesis is rendered more likely by the OCT and CS increases of about five and twofold, respectively. The liver seems to work differently and its role, if any, in nitrogen excretion is probably of little relevance.

The metabolic profile of the marble swamp eel under semi-aestivation would be typical of fasting if acidemia had been observed; however, blood pH remained constant. Direct correlation between aestivation and fasting, as reported by Janssens (1964) and DeLaney *et al.* (1977), seems not probable in the case of *S. marmoratus*. Nevertheless, starvation-related metabolites may possibly act as signals for aestivation. For instance, the rise of urea-synthesis enzymes means increase of ornithine and arginine, intermediates believed to increase polyamine concentrations. Such molecules play a role in the regulation of many protein syntheses (Aoyama, 1989, 1990). Moreover, the hyperuricemia observed in the first 15 days of aestivation can result from overactive metabolism of informational molecules during the initial phases of metabolic adjustments. New evidence must be found regarding the role of small molecules in general metabolism, such as those mentioned above, in which different metabolic processes are triggered and/or adjusted in response to environmental factors.

In conclusion, catabolism of amino acids and lipids plays an important role in the process of

supplying energy for the semi-aestivated *S. marmoratus*. Fat stores of the animals used were depleted in 45 days and aerobic metabolism was not suppressed. The reduction of lactate in every tested tissue, the small amount of urea synthesized after 45 days of semi-aestivation, and the animals' general torpor were suggestive of very slow metabolism. The water balance was not disturbed and ammonia excretion was shifted toward urea synthesis and its waste. Regarding intermediary metabolism, a role is being proposed for small molecules in assisting the physiological adjustments of *S. marmoratus* in the semi-aestivation process.

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