Vol.49, n. 5 : pp. 825-833, September 2006
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

L-Arginine Metabolism in Mitochondria Isolated From the Liver of Antarctic Fish *Notothenia rossii* and N*otothenia neglecta*

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ABSTRACT

The arginase tissue distribution, the biochemical properties of the argininolytic system and the subcellular localization of the enzymes carbamoylphosphate synthetase, ornithinecarbamoyl transferase, glutamine synthetase and arginase in Antarctic fish, N. neglecta and N. rossii were the main aims of the present work. The tissue with highest argininolytic activity was the kidney distal portion amounting as much as four times the specific activity of the hepatic tissue. Arginase and ornithine carbamoyltransferase were found as mitochondrial enzymes, while glutamine synthetase and carbamoylphosphate synthetase were found as cytosolic enzymes. Argininolytic assays with isolated mitochondria gave values of K_{mapp} for the hydrolysis of arginine 2 to 3.5 times higher than the values found for the K_m with mitochondrial extracts. The effect of Mn^{2+} on the argininolytic activity displayed by isolated mitochondria and mitochondrial extracts, in reaction conditions near the physiological ones showed that membranes were fundamentally involved in the control of L-arginine metabolism.

Key Words: Antarctic fish, arginine, arginase, urea cycle, *N. neglecta, N. rossii*

INTRODUCTION

The L-arginine metabolism is closely related to the ureogenic activity in animals and excretion of urea, probably a prerequisite for the life in terrestrial environments, which display intermittent disposability of water (Hird, 1986; Mommsen and Walsh, 1989). The maintenance of cellular L-arginine levels is also essencial as precursor for the synthesis of proteins, polyamines, creatine, proline, glutamate and nitric oxide (Wu and Morris, 1998). Thus, the biological membranes have a central role in L-arginine subcellular distribution. Yeast cells have high capacity to concentrate L-arginine in vacuolar structure. The transport at the vacuolar membranes in *Neurospora crassa* and *Saccharomyces cerevisiae* is ATP dependent on K_m values of 0.40mmol/l and 0.65mmol/l repectively (Zeres et al., 1986; Sato et al., 1984 a,b). However, the *N. crassa* transport of L-arginine in mitochondria is passive, with a K_m of 6.5mmol/l (Yu and Weiss, 1992).

L-Arginine hydrolysis is catalyzed by a restricted group of Mn^{2+} dependent enzymes, the arginases, which are widely spread in the biosphere. In mammals, two arginase isoenzymes, type I and type II, occur displaying similar physicochemical properties at different subcellular sites. Type I arginase is found at the hepatocytes cytosol, actively associated with the urea cycle. Type II arginase is a mitochondrial enzyme found in

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extrahepatic tissues with very little or almost no meaning with respect to the liver metabolism (Jenkinson et al., 1996). The physiological meaning of type II arginase is not quite clear, probably being involved with the maintenance of L-arginine at intracellular levels in extrahepatic tissues for the control of polyamines, GABA, nitric oxide, proline and glutamate metabolism (Russel and McVicker 1972; Johnson and Roberts, 1984; Verma and Boutwell, 1981; Yip and Knox, 1972; Daghigh et al., 1994).

Normally, fish arginases are present in the mitochondrial fraction, except in dipnoic fishes (lungfish) that possess cytosolic arginase Mommsen and Walsh (1989). However, studies with Batrachoididae fish showed that arginase was equally distributed in both mitochondria and cytosolic fractions, amounting around fifty percent of activity in each compartment. Furthermore, both arginase fractions displayed identical physicochemical properties in ion exchange chromatography and in polyacrylamide gel electrophoresis (PAGE) without denaturation conditions and thus, corroborating the assumption that both were a single enzyme (Anderson and Walsh, 1995).

The low ureogenic potential of the enzymes present in the Krebs-Henseleit cycle has been established by studies with Antarctic fishes from McMurdo Bay (Raymond and DeVries, 1998) and from Admiralty Bay (Rodrigues et al., 2002). However, it has been found that the catalytic activitiy of L-arginase is far above the catalytic activity of the other enzymes of the Krebs-Henseleit cycle. Furthermore, an interesting observation was that the catalytic activity concentration of hepatic arginase from *Notothenia rossii* was 10 times higher than that of *Notothenia neglecta.*

Thus, the main aim of the present work was to study the tissue metabolism of L-arginine in the Antarctic fish *N. neglecta* and *N. rossii* by analysing the levels of arginase and its kinetic properties in mitochondrial suspension and extract near fish physiological temperature, trying to understand the role of mitochondria membranes in the transport of L-arginine, as well as the subcellular site of the main enzymes of the Krebs-Henseleit cycle.

MATERIAL AND METHODS

Mature *Notothenia rossii* and *neglecta* were captured near the Brazilian Antarctic Station Comandante Ferraz at Admiralty Bay, King George Island, South Shetlands, Antarctic. Fish weighting 310 to 450g were maintained without foods at 0^oC in an aquarium in a proportion of 1 fish/100 l of sea water under constant aeration. The period of time between the capture of the fish and the sampling was 48 to 72h. Freshly excised tissues were homogenized in the proportion of 1g for 10ml of a HEPES buffer 20mmol/l pH 7.4 containing trimethylamine N-oxide 1mmol/l, KH_2PO_4, 5mmol/l, EDTA 0.5mmol/l and sucrose 250mmol/l (compartmentalization buffer) as described by Polez et al. (1998).

The homogenates were spun down for 10 min at 1,500xg in a refrigerated centrifuge and the supernatants were used for enzymatic assay. For the preparation of the mitochondrial fraction, the supernatants were spun down 10 min at 14,000xg and the sediment was washed twice with the compartmentalization buffer. For the preparation of the mitochondrial extract, part of the mitochondrial sediment obtained as above was suspended in the same buffer without EDTA but with Triton X-100 0.1% (v/v), sonicated twice with 30 sec strokes for the disruption of the mitochondria and then spun down 10 min at 14,000xg the supernatant was used for kinectic studies. All the kinectic studies were carried out at 0^oC.

The mitochondria integrity was evaluated by means of the assay of glutamate dehydrogenase (GDH). The cytosolic contamination of the mitochondrial fractions was evaluated by the assay of lactate dehydrogenase (LDH). The assays of LDH and GDH activities were carried out as described by Vorhaben and Campbell (1972) and Ciardello et al. (1997), respectively. The combined actvities of carbamoylphosphate synthetase I and III (CPS-I and CPS-III) were assayed in 1ml of the HEPES buffer 50mmol/l, pH 8.0 containing (mmol/l) ATP 20, glutamine 10, NH_4Cl 100, L-ornithine 10, $NaHCO_3$ 10, $MgSO_4$ 24, N-acetylglutamic acid (AGA) 5 and ornithine carbamoyl transferase (OTC) 1U/ml. The reaction was suspended by adding of 200µl of trichloroacetic acid (TCA, 70% w/v) and the content of the tube were centrifuged for 10 min at 14,000xg and the supernatant was used for

citrulline determination according Bergmeyer (1983).

Ornithine carbamoyl transferase was analysed in 1ml triethanolamine (TEA, 200mmol/l, pH 7.6), L-ornithine and carbamoyl phosphate 5mmol/l each. The reaction was stopped by the addition 500µl of 5mol/l perchloric acid and kept for 10 min in an ice bath and then spun down for 10 min at 14,000xg. The citrulline formation was assayed according to Bergmayer (1983). Glutamine synthetase (GS) was analyzed in 1ml of TRIS-acetate buffer (80mmol/l, pH 7.4), containing (mmol/l) hydroxylamine 40, $MgSO_4$ 20, sodium glutamate 80, ATP 10, phosphoenolpyruvate 10, pyruvate kinase 0.6U and β-mercaptoethanol 20mmol/l. The reaction was stopped adding 1,5 ml of 0,37M ferric chloride in 0,67 M HCl and 0,2 M trichloroacetic acid, and spun down for 10 min at 14,000xg to remove the denatured protein (Pamijans et al., 1962). The tissue level arginase activity was determined in glycine buffer (60mmol/l pH 9.5) containing (mmol/l) L-arginine 250 and $MnCl_2$ 5 and in TRIS-HCl (80 pH 7.4) containing L-arginine 250 and $MnCl_2$ 5mmol/l respesctivelv. The urea originated from this reaction was assayed by the method of Geyer and Dabich (1971).

All enzymes activities were expressed in International Units (µmol/min). The protein content was estimated by the method of Lowry et al. (1951).

RESULTS AND DISCUSSION

The relative distribution of arginase activity in *N. neglecta* tissues is shown in Fig. 1. The argininolytic activity in this Antarctic fish was high in the kidney's distal portion and liver than other tissues. Hunter (1929) reported that liver was the main organic territory for the genetic expression of arginase. Also, Campbell (1961) found that in *Mustellus canis*, arginase level was as high as 1.12 and 0.67 U/mg protein in the liver and kidney respectively, when kept under saturating conditions of substrate in pH 9.5 and at 20°C. Arginase activity values in *N. neglecta*'s liver, whole kidney and its distal portion at 0°C in substrate saturation condition were 4.8±3.0, 19.2±4.6 and 39±26.0mU/mg respectively. These assays were carrried out at a non-physiological pH 9.5, resulting in an activity values 4 to 6 times higher than that found at pH 7.4. Felski et al. (1998) observed high levels of arginase in kidney than in liver of the different species of teleost fish.

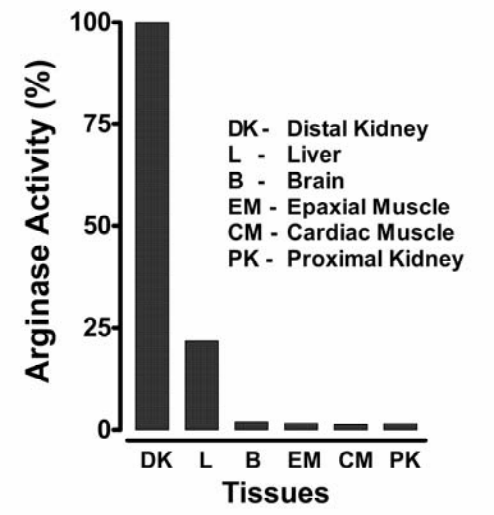

Figure 1 - Arginase specific activity percentual levels in *N. neglecta* tissues (n=8). The activities were assayed in cell free homogenates after spinning down at 1,500xg. Reaction medium: glycine, 50mmol/l, pH 9.5, L-arginine 250mmol/l and $MnCl_2$ 5mmol/l.

The activity of arginase found in the kidney of *Cyprinus carpio* was 4 times higher than that found in the liver while in the kidney of *Carassius auratus*, the activity of arginase was 50 times higher than that found in liver. In spite of the fact that the fish species studied by Felski et al. (1998) were neither ureotelic nor ureosmotic ones, they showed different levels of tissue arginase, a physiological condition that could be due to the maintenance of adequate levels of tissues L-arginine.

Results regarding the subcellular distribution of liver enzymes from the urea and the glutamine metabolism in *N. neglecta* are shown in Fig. 2. Arginase and OTC showed to be the enzymes from the mitochondrial cell compartment, while GS and CPS were prevalent in the cytosol of hepatic cells. The specific activities of CPS, OTC and arginase at the mitosol of hepatic cells from *N. neglecta* and *N. rossii* are shown in Table 1.

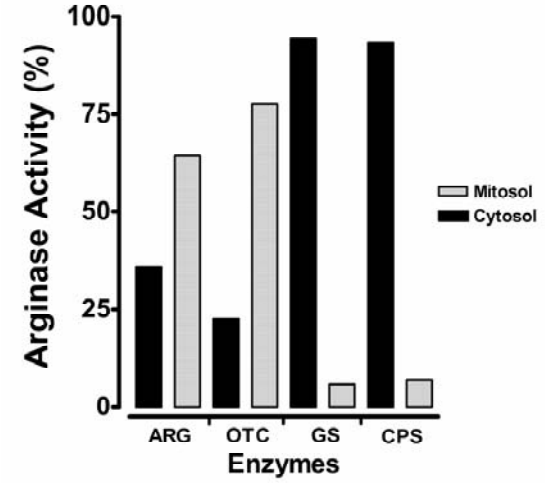

Figure 2 - Subcellular localization of enzymes from the urea and glutamine metabolisms in *N. neglecta* liver (n=8). Lactate dehydrogenase (LDH) and glutamine dehydrogenase (GDH) were used as markers of the subcellular fractions. LDH activity was 98.8% at the cytosol and 1.2% at the mitosol and GDH was 76.7% at the mitosol and 23.3 % at the cytosol. ARG, arginase; OTC, ornithine transcarbamoylase; GS, glutamine synthetase; CPS, carbamoylphosphate synthetase.

Along the evolution of vertebrates, both arginase and CPS-III were preserved at the liver mitochondrial fraction in teleost and elasmobranch fish. Conservation of CPS-III to CPS-I seemed to be happened during the step of evolution that was directed towards the origin of the lungfish, a dipnoic fish (Mommsen and Walsh, 1989). In view of the fact that a few species of fish with low capability for urea synthesis expressed mitochondrial GS, its presence in the mitochondrial fraction was of particular importance in ureosmotic fish displaying a functional urea cycle where glutamine acted as substrate for the CPS-III reaction.

Table 1 - Specific activity of arginase, ornithine transcarbamoilase (OTC) and carbamoylphosphate synthetase (CPS) in mitochondrial extracts from the liver of Antarctic fish *N. neglecta* (n=8) and *N. rossii* (n=7). The activities were assayed at pH 7.4 and at 0°C.

Enzymes	**Specific Activity ± SD (mU/mg protein)**	
	N. neglecta	*N. rossii*
Arginase	19.46±10.12	89.20±45.28
OTC	0.10±0.044	0.90±0.43
CPS	0.017±0.006	0.021±0.002

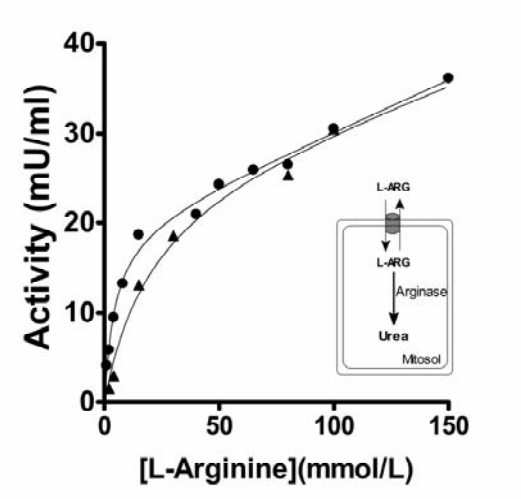

Figure 3 - Kinetic parameters involving the effect of L-arginine on the argininolytic activity. Aliquot of *N. neglecta* liver mitochondrial extracts (-●-) and suspension (-▲-) were incubated in compartmentalization buffer without EDTA, pH 7.4 containing $MnCl_2$ 0.5mmol/l. The reactions were carried out at a temperature of 0ºC.

The low specific activity of CPS and OTC enzymes in Antarctic fish from McMurdo Bay (*D. mawsoni, G. acuticeps, N. angustata, L. dearborni)* studied by Raymond and DeVries (1998) as well as the the values shown in Table 1 for fish from Admiralty Bay - King George Island, are the characteristics of fish displaying low ureogenic capacity.

Nevertheless, very near in the phylogenetic range, *N. rossii* displayed 5 and 9 times higher levels of OTC and arginase respectively than that found in *N. neglecta*, a fact regarded as the metabolic needs congenital to the feeding behavior of these species. Considering the central role displayed by arginase in maintaining adequate levels of L-arginine in tissues and biological fluids, these experiments were conducted at pH and temperature near the fish physiological condition. On the other hand, the subcellular sites of arginases from Antarctic fish, as well as the hepatic tissue argininolytic activity encompass also L-arginine transportation through the mitochondrial membrane. Furthermore, the subcellular compartmentalization of arginase and the disponibility of Mn^{2+} in the mitochondrial spaces may represent an important site for the metabolic control of L-arginine.

The L-arginine K_{mapp} values assayed with suspensions of liver mitochondria from *N. neglecta* and *N. rossii* were 8.42±3.95 and 9.16±0.94mmol/l, while the K_m values assayed with mitochondria extracts were 2.40±0.55 and 4.46±0.53mmol/l, respectively (Fig. 3). Arginases from teleost fish displayed K_m values for L-arginine around 11.6mmol/l at pH 9.5, while arginases from elasmobranch fish displayed K_m values of 1.2mmol/l at pH 9.8 (Jenkinson et al., 1996). Considering that the optimum pH for arginases was around 9.5, most of the kinetic data so far obtained were carried out in reaction media with pH values away from the physiological band of living organisms (Campbell, 1961; Kadowaki and Nesheim, 1979; Reczkowski and Ash, 1994; Jenkinson et al., 1996).

Another important aspect to be considered is in regard to the subcellular site of arginases and their distribution along the phylogenetic rank. In this sense, the determination of the kinetic constants of mitochondria arginase in tissue homogenates may be a most valuable source of information in regard to the transport mechanism of L-arginine through the mitochondrial membrane. Experiments with liver homogenates from the elasmobranch *Mustelus canis* showed K_m = 8.11mmol/l in a medium containing glycine at pH 9.5, which was far above the values described so far for elasmobranches (Campbell, 1961).

Carvajal et al. (1987) found values of K_m of 9.1mmol/l at pH 7.6 and 22.5mmol/l at pH 9.6 at 37º C in a partially purified liver arginase preparation from the teleost fish *Genypterus maculatus*. In liver arginase preparation from *Merlucius gayl*, Carvajal et al. (1989) found K_m

values of 1.7 and 10.3mmol/l at pH 9.5 and 7.5, respectively without alterations at the temperatures between 9 and 37°C. They also pointed out that site of arginase in the mitochondria matrix and their respective kinetic properties might avoid the indiscriminate hydrolysis of arginine and controled the use of this aminoacid as a source of energy at the *M. gayl* liver.

Comparing the kinetic data of arginases from *N. neglecta* and *N. rossii*, was K_{mapp} 3.6 and 2.0 times higher than that of the K_m values, respectively. The differences between K_m and K_{mapp} determinations take place in the access of arginine towards the argininolytic system, that is, the K_{mapp} at the mitochondrial membrane may play an important physiological role in the control of arginine levels in cell.

In order to elucidade the role of the mitochondrial membrane in the arginolytic process, an experiment was carried out to establish the aproximate time of L-arginine transport across the mitochondrial membrane (Fig. 4). The time of arginine transport towards the mitochondrial spaces limited the arginase substrate disposability and the consequent urea synthesis, as shown in Fig. 4. Urea formation was linear in the system containing mitochondria extract, while in the system containing mitochondrial suspension, it became linear only 20 minutes after the begining of the experiment.

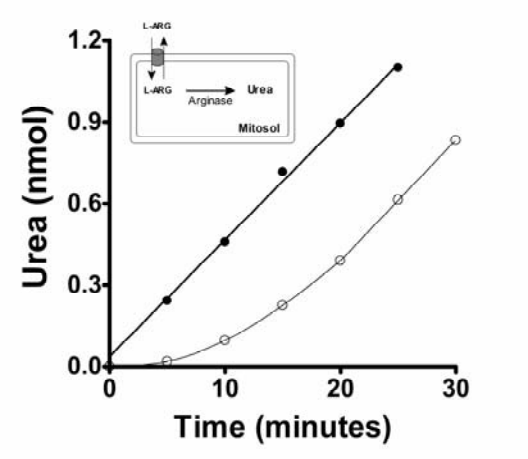

Figure 4 - Time course of L-arginine transport. Samples of *N. neglecta* liver mitochondrial fraction (-o-o-) and extracts (-•-•-) were incubated in compartmentalization buffer without EDTA, pH 7.4, containing L-arginine 100mmol/l and $MnCl_2$ 5mmol/l. The reactions were carried out at a temperature of 0ºC near fish physiological conditions.

Results showed that the arginine metabolism in mitochondrial suspensions involved also the arginine transport through the mitochondrial membrane, a process that reached an equilibrium establishing a stationary state of L-arginine concentration between both the outer and inner mitochondrial compartments. Thus, the differences found in the kinetic behavior of the preparations in Fig. 4 suggested that the mitochondrial membranes displayed a fundamental role in the L-arginine access towards the inner mitochondrial spaces. This observation was supported by the 2.0 to 3.6 difference found between the K_{mapp} established for the mitochondrial suspension and the K_m found for the mitochondrial extracts, suggesting that the mitochondrial membranes displayed an important role in the access of L-arginine towards the intramitochondrial spaces.

Studies by Rodrigues et al. (2004) on the comparative aspects of L-arginine metabolism carried out with the Antarctic fishes *N. neglecta* and *N. rossii*, and the tropical fish grouper (*Epinephelus marginatus*) showed that arginase K_m assayed in both mitochondrial suspensions and mitochondrial extracts gave lower values in tropical fishes preparations than the ones found in the Antarctic ones. The effect of manganes ion on the activity of the argininolytic system in Antarctic fish (*N. neglecta* and *N. rossii*) was evaluated in mitochondrial suspension and extacts (Fig. 5). It is known that the catalytic activity of arginases from different species depends on the presence of a divalent ion, especially Mn^{2+} that acts as an

activator and stabilizer of the enzyme structure (Kuhn, 1991). In the cytosolic fraction of rat liver, Maggini et al. (1992) described the presence of arginase with two sites for Mn^{2+}. It was postulated that arginine and Mn^{2+} in concentrations near the physiological ones could control ureogenesis *in vivo*.

In arginine concentrations near the saturation level and at pH and temperature near the physiological condition, the argininolytic systems present in mitochondrial suspensions and extracts from the liver of *N. neglecta* and *N. rossii* were activated by Mn^{2+} below the 500μmol/l. However, Mn^{2+} above the 500μmol/l, the argininolytic system present in mitochondrial extracts was inhibited while the system containing mitochondria was activated. This showed the role of the mitochondria membranes in the control of argininolytic activity in the liver of Antarctic fish. Studies on the mechanism of action of Mn^{2+} on the arginase activity as well as maintainance of the protein structure and the argininolytic process, have contributed for the understanding of L-arginine metabolism cell and tissues (Reczkowski and Ash, 1994; Green, et al., 1991; Maggini et al., 1992).

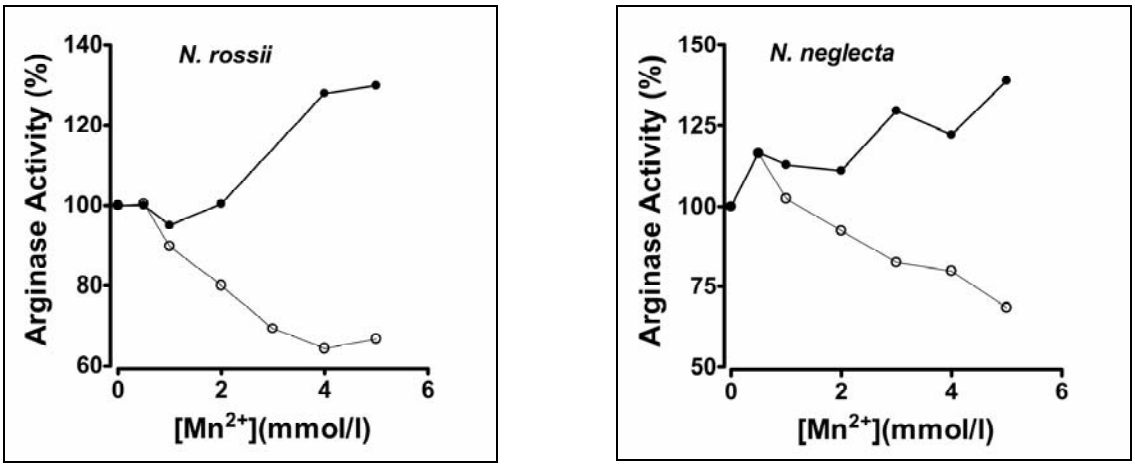

Figure 5 - The effect of the Mn^{2+} ion on the argininolytic activity. Samples of liver mitochondrial fraction (-•-•-) and extracts (-o-o-) were incubated in compartmentalization buffer without EDTA, pH 7.4, containing L-arginine 15mmol/l. The reactions were carried out at the temperature of 0ºC.

Most of these kinectic studies were carried out with arginases purified from different species and the experiments were not carried out always near the physiological conditions. On the other hand, the subcellular distribution of enzymes in the nitrogen metabolism, in especially arginase, has labeled the evolution of living beings as a reflex of a probable strategy of metabolic adaptation. In this sense, the mitochondrial localization of arginases deserve a special attention with regard to the possible role of mitochondrial membranes in the control of argininolytic process and to the tissue needs of this amino acid towards protein synthesis and of other nitrogen compounds. Present results showed the importance of the mitochondrial membranes in *N. neglecta* and *N. rossii* hepatic arginolysis control near the physiological conditions.

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Received: December 17, 2004;
Revised: October 06, 2005;
Accepted: April 04, 2006.