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A COMPARATIVE STUDY OF HEPATIC MITOCHONDRIAL OXYGEN CONSUMPTION IN FOUR VERTEBRATES BY USING CLARK-TYPE ELECTRODE

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The present study was undertaken to establish a comparative account on hepatic mitochondrial oxygen consumption of *Clarias gariepinus* (fish), *Bufo melanostictus* (amphibian), *Gallus gallus* (bird) and *Rattus norvegicus* (mammal) and to correlate it with their specific metabolic rate (SMR). Mitochondrial oxygen consumption was measured with a Clarke-type electrode with succinate and pyruvate/malate as substrates. ADP was used to start state-III respiration. The results show that rats and chickens have higher oxygen consumption rate than that of fish and toads. Similarly, a species and substrate specific difference was also noticed in P/O (phosphate utilized per oxygen atom) ratio and respiratory control index. In case of rat, a significant negative correlation was noticed between P/O ratio and SMR with succinate as substrate. It is surmised that the observed difference in the mitochondrial respiration and P/O ratio in the above vertebrates is due to the difference in their metabolic activities.

Keywords: Mitochondria – state-III and -IV respiration – Respiratory Control Index – P/O ratio – specific metabolic rate

INTRODUCTION

In general, 90% of total oxygen inhaled is taken by mitochondria out of which 95–98% is completely reduced to water during oxidative phosphorylation [10]. The rate at which a resting animal carries out metabolism is determined mainly by its phylogenetic group, thyroid-hormone level, temperature and body size, and the standard metabolic rate (M) of vertebrates which depends on size according to the equation $M = aW^{075}$, where W is body mass. The value of the elevation constant, a, is about 4–5-fold higher in homeothermic animals (mammals and birds) than it is in reptiles and other poikilotherms. Therefore, a resting mammal consumes oxygen 4–5 times as fast as a reptile of the same body mass, at the same body temperature [15, 21, 26].

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Due to its respiratory activity, mitochondria are the important site for the consequences of oxygen metabolism [25, 26]. It is well established that both respiration and its consequences have direct relation with the metabolic state of different tissues of same animal and same tissue of different animals [17, 24, 32]. In this context, very few studies have been reported on animals other than mammals as far as mitochondrial oxygen consumption measured with a Clark-type electrode is concerned. Using the same method, a comparative inter- or intra-specific investigation among vertebrates gives many clues to understand the physiological significance of the measured parameters [10, 32]. Comparative studies within the species at morphological level [22, 23], tissue concentration of metals at biochemical level [9], mitochondrial level [17] or mitochondrial 16 S and 12 S rRNA marker [11] or nucleotide sequence [20] at molecular level have a lot of significance for establishment of phylogenetic relationships among the animals. Although most of the work regarding oxygen consumption by mitochondria using oxygraph (Clark-type electrode) is confined to rats in general, not much information is available on other classes of vertebrates, particularly in case of fish. Therefore, in the present study an attempt was made to compare the oxygen consumption by liver mitochondria of different vertebrates using oxygraph. For comparative purpose, oxygen consumption was investigated in hepatic mitochondria of fish, amphibian, avian and mammalian models.

MATERIALS AND METHODS

Animals

Catfishes *(Clarias gariepinus)* were purchased from the local venders, disinfected by treatment with potassium permanganate solution (500 ppm, 3 min) and kept in the laboratory water reservoir for 7 days for acclimatization. They were fed with the commercial fish feed containing rice bran and groundnut oil cake in equal proportion. The animals ($n = 7$) of 166 ± 33 g body weight were used for the study. Indian common toads *(Bufo melanostictus)* were collected from a specific area of Utkal University, Bhubaneswar campus before a day of their sacrifice. The animals ($n = 7$) of 60 ± 6 g body weight were used for the study. Commercial broiler chickens (*Gallus gallus,* $n = 7$) of approximately 1.5 ± 0.11 kg body weight represented the avian group and were from Sahid Broilers, Bhubaneswar, India. The birds were fed with the commercial broiler feeds once a day. Fresh liver samples were collected immediately after sacrifice of the birds from the slaughterhouse. Wistar strain rats *(Rattus norvegicus)* were obtained from National Institute of Nutrition, Hyderabad, India. They were kept in polypropylene cages ($430 \times 270 \times 150$ mm^3) with stainless steel hopper in a well-ventilated room with 12 h light: 12 h dark cycle. Rats were maintained in laboratory as described earlier [29, 30]. Tap water was supplied *ad libitum.* Rats ($n = 5$) of body weight 121 ± 12 g were used for the experiment. All experiments were conducted under the guidance of Institutional Animal Ethics Committee as approved by

Committee for the Purpose of Supervision and Experimentation on Animals (CPCSEA), Govt. of India. All care was taken to minimize the number of animals used and their suffering.

Isolation of mitochondria

All the animals except chicks were sacrificed after overnight fasting for experimentation so as to deplete hepatic fat load. In chick no fasting was done since their livers were directly collected from the slaughterhouse. The other three groups of animals were sacrificed by decapitation. After sacrifice of the animals, livers were immediately removed by a mid-ventral incision, washed in ice-cold physiological saline solution. The mitochondria were isolated from the liver according to Rusyniak et al. [28] with a little modification. The liver was washed in ice-cold normal saline to remove contaminating blood followed by washing with ice-cold mitochondria isolation buffer (IB) containing 210 mM mannitol, 10 mM sucrose, 5 mM HEPES and 1 mM EGTA (pH 7.4). The tissue was then minced and washed again with IB. A 10% (w/v) homogenate was prepared in IB with the help of a Potter–Elvehjem type motor-driven glass-teflon homogenizer at a speed of 250 rpm with three up and down strokes. The homogenate was centrifuged at $600 \times g$ for 10 min at 4 °C to separate the nuclear fraction and tissue debris in a refrigerated high speed centrifuge (REMI, Mumbai, India, Model no.-C 24). The supernatant was then centrifuged at $10{,}000 \times g$ for 10 min at 4 °C to separate the mitochondrial fraction. The mitochondrial pellet was mixed with 2 mg of fatty acid free BSA per gram liver tissue in IB. In chick, the amount of BSA added was 3 mg per g of liver tissue. Mitochondrial pellet was washed and dissolved in respiration buffer (RB) containing 125 mM KCl, 4 mM KH_2PO_4, and 10 mM MOPS (pH = 7.4). Protein concentration of mitochondrial sample was measured by Bradford reagent [4], as per manufacture's protocol (Biogene, Reagents, USA) using BSA as standard.

Measurement of mitochondrial respiration and specific metabolic rate (SMR)

Mitochondrial oxygen consumption measurements were finished within 1 h of pellet isolation by using a Clark-type electrode (Hansatech DW1; Hansatech Instrument Ltd., King's Lynn, Norfolk, England) with Oxygraph Plus software; Version 2.1. The respiration chamber was connected with a water circulator to maintain constant temperature of 25 °C. The reaction was carried out at a constant stirrer speed at 100 rpm in 1.5 ml chamber volume with ~1 mg protein. Recording of oxygen consumption by isolated mitochondria was started by adding calculated amount of respiration buffer into the chamber followed by mitochondria sample. Then mitochondria (1 mg/100–200 µl) were added into the chamber followed by 10 µl succinate (10 mM) or 5 µl each of pyruvate (5 mM) and malate (5 mM) as substrates through a Hamilton syringe

at 10 sec time interval. St-III respiration was initiated by adding 5 µl ADP (250 nmoles) into the respiration chamber. After consuming all the ADP, st-IV respiration was achieved. Concentration of ADP was increased wherever required and Respiratory Control Index (RCI) and P/O ratio were calculated. RCI was calculated by dividing state-III respiration rate by state-IV respiration rate. ADP molecules utilized to oxygen uptake (P/O) ratio were calculated manually from the oxygraph by dividing the ADP molecules utilized with the number of atomic oxygen consumed in state-III respiration. Specific metabolic rate (SMR) of the animals was calculated using Kleiber's equation 16: SMR ($cal \cdot g^{-1} \cdot day^{-1}$) = 393 (g body weight)$^{-0.25}$ [18].

Statistics

Analysis of variance followed by Duncan's New Multiple Range test was carried out to find out the difference in mean values among the species at $p < 0.05$ level. Linear regression analysis and correlation co-efficient were calculated taking SMR against respiration parameters.

RESULTS AND DISCUSSION

The results of the present study clearly suggest that the rate of oxygen consumption by the mitochondria at complex-I and complex-II was comparatively higher in homeotherms (bird and mammal) in comparison to poikilotherms (amphibia and fish). RCI value for complex-I and -II in case of fish was higher than that of toads. The lower RCI value suggests that mitochondria were in uncoupled state [29, 30]. P/O ratio in case of fish was twice higher in complex-II than complex-I. The situation was just reverse in case of toads (Table 1). Such differences in P/O ratio may be due to different ecological adaptations of animals. Guderley et al. [14] compared the relationship between molecular composition of muscle mitochondria and its oxidative capacity in rat *(Rattus norvegicus),* the cane toad *(Bufo marinus)* and the bearded dragon lizard *(Pogona vitticeps)* and concluded that molecular composition of mitochondria of different vertebrates contribute to their mitochondrial capacities. The authors found that oxidative capacity was higher in rats and cane toad in comparison to bearded dragon lizard. In another study, using the same specimens, the data obtained by Hulbert et al. [17] are in good agreement with our results.

In *Anguilla anguilla,* P/O ratio was reported to be 1.31 ± 0.08 and 2.52 ± 0.04; for succinate and pyruvate/malate as substrate at pressure 0.1 MPa (control pressure), respectively, whereas for muscle mitochondria these values changed to 1.43 ± 0.07 and 2.87 ± 0.05 when the pressure was elevated to 10.1 MPa [31]. In the present study, in *Clarias gariepinus,* P/O ratio and RCI value did not vary whether substrate used is pyruvate/malate or succinate. However, RCI value for *Clarias gariepinus* is similar to that of *Anguilla anguilla*. In toadfish, *Opsanus beta* P/O ratio and RCI value for brain mitochondria were reported as 2.58 and 4.6, respectively [33]. Such differences

Table 1
Hepatic mitochondrial state-III and state-IV respiration (nmol oxygen/mg protein), respiratory control index (RCI), phosphate to oxygen atom ratio (P/O) and specific metabolic rate (SMR) in four vertebrates

Animals	State-IV		State-III		RCI		P/O		SMR
	succinate	pyruvate/ malate	succinate	pyruvate /malate	succinate	pyruvate/ malate	succinate	pyruvate/ malate	
Fish (n = 7)	7.01± 2.11[a]	5.35± 1.58[a]	17.05± 7.07[a,b]	12.52± 5.37[a,b]	2.41± 0.43[a]	2.23± 0.48[a]	1.82± 0.58[a]	2.21± 0.80[a]	110.46± 5.88[a]
Amphibia (n = 7)	7.57± 1.66[a]	6.48± 2.21[a]	11.14± 1.99[b]	9.42± 3.35[b]	1.47± 0.09[b]	1.46± 0.30[b]	2.47± 0.71[a]	1.52± 0.27[b]	141.31± 3.57[b]
Bird (n = 7)	13.13± 6.05[b]	12.67± 5.04[b]	23.92± 10.66[a,c]	19.07± 8.28[a,c]	1.85± 0.37[c]	1.51± 0.35[b]	2.15± 0.51[a]	2.24± 0.69[a]	65.06± 1.28[c]
Mammal (n = 5)	27.61± 9.79[c]	19.72± 4.54[c]	67.17± 17.06[d]	31.31± 11.77[d]	2.49± 0.24[a,d]	1.71± 0.22[b]	1.46± 0.13[b]	1.52± 0.32[b]	118.57± 2.71[d]

Succinate (10 mM) and pyruvate/malate (5 mM each) were used as substrate with 250 nmoles ADP. Data are expressed as mean ± S.D. of number of animals given in parentheses taken in triplicates. Different superscripts show statistical different within the groups at $P \leq 0.05$ level.

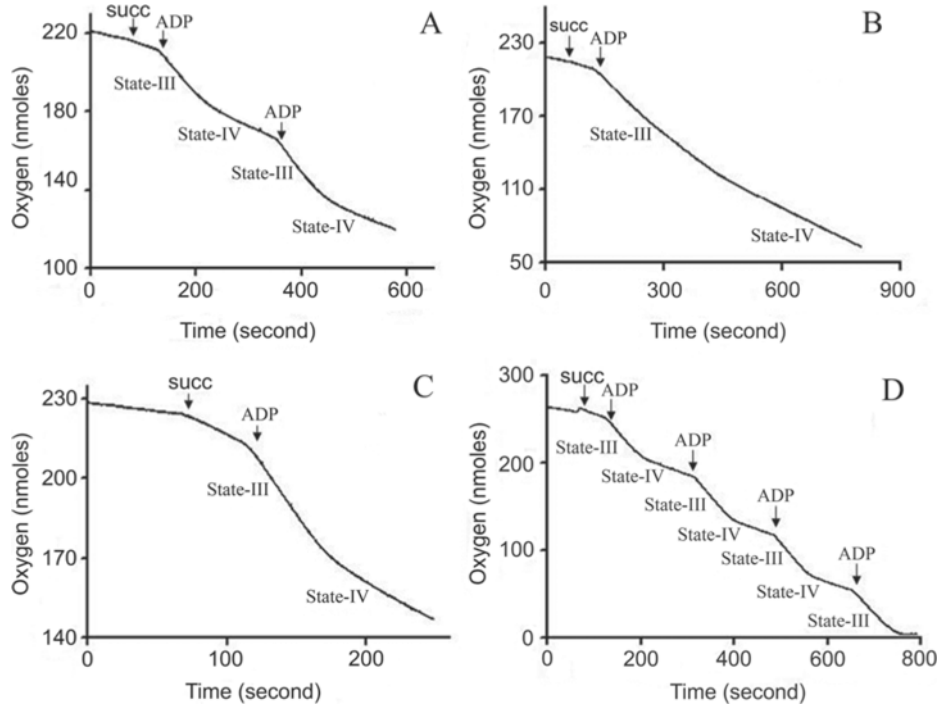

Fig. 1. Four representative oxygraphs showing state-III and state-IV respiration in hepatic mitochondria of fish, *Clarias gariepinus* (A), amphibian, *Bufo melanostictus* (B), bird, *Gallus gallus* (C) and mammal, *Rattus norvegicus* (D) with 10 mM succinate as substrate and 250 nmoles ADP

in P/O ratio and RCI values for mitochondria of different fish species may be due to tissue specificity or may also be due to species specificity since all the three species taken for comparison were from different habitats.

The rate of oxygen consumption (both state-III and state-IV) was the highest in mammal followed by bird, whereas, both toad and fish showed lower values irrespective of the substrate used. P/O ratio was the lowest in mammals (Table 1 and Fig. 1). Oxygen consumption is directly related to electron transport in mitochondria, which is coupled with production of ATP by oxidative phosporylation [19]. Similarly, the rate of ATP generation (St-III × P/O, nmol/min/mg pr) [25] was the highest in mammals (98.07 via succinate, 47.59 via pyruvate: malate) followed by birds (51.43 via succinate, 42.72 via pyruvate: malate), fishes (31.03 via succinate, 27.67 via pyruvate: malate) and amphibians (27.52 via succinate, 14.32 via pyruvate: malate). It indicates the phylogenetic metabolic depression order in the above sequence. Brand et al. [6] have opined that the non-linear relationship between respiration rate and proton motive force in isolated mitochondria is entirely by iP-dependent changes and in the proton conductance of the mitochondrial inner membrane and is not caused by redox slip in the proton pumps. They have reported that mitochondrial proton leak occurs

in intact cells and tissues accounting for 33 (±7)% of the mitochondrial respiration rate of isolated hepatocytes suggesting that heat production may be an important function in the proton leak in homeotherms [6]. The mitochondrial proton conductance in isolated mitochondria and in hepatocytes is greatly modulated by thyroid hormone [13]. It is also influenced by nitrogenous base metabolizing enzymes [5], phylogeny [16] and body mass [8]. Usually, the reactions of ATP turnover rate change in parallel to respiration rate so that the coupling ratio is not greatly affected [3, 7]. Akhmerov [1] suggested that mitochondria from frog liver and heart were less leaky to protons than mitochondria from the rat. Probably that is why toads showed maximum SMR among the vertebrate groups studied (Table 1). In case of rats, a significant negative linear correlation was observed taking SMR against P/O ratio with succinate as substrate ($y = -0.039x + 6.103$, $r = -0.9099$, data not given). However, in rest of the animal groups studied, no significant correlation between SMR and P/O ratio was found indicating less leakage. Since both fish and amphibian are poikilotherms, it is obvious that their mitochondria are less leaky in comparison to the endothermic animal such as rat [12]. In the present study, it is surprising that in case of the endothermic avian species no such correlation was found, which may be due to their in captive sedentary life and transport of liver samples from the slaughter house. We suggest that the observed difference could be due to its restricted metabolism rather than its phylogenetic development in terms of endothermy. Therefore, the captive avian group may not be taken as a representative group for comparative purposes in mitochondrial respiration studies. Albeit, the observed respiratory parameters are in conformity to those reported by Allred and Roehrig [2] and the results are far below the rate of oxygen consumption by pigeon muscle mitochondria which is metabolically more active [27]. Furthermore, the animal groups studied here differ significantly in their body weight, organ weight, organ to body weight ratio as well as preferred body temperature (data not given) which may be another reason for the observed variations [6].

In conclusion, the results of the present study suggest that the differences in the mitochondrial respiration and ATP generation in major vertebrates, i.e. mammal, bird, amphibian and fish are due to the difference in their metabolic activity.

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REFERENCES

1. Akhmerov, R. N. (1986) Qualitative difference in mitochondria of endothermic and ectothermic animals. *FEBS Lett. 198,* 251–255.
2. Allred, J. B., Roehrig, K. L. (1969) Respiration of isolated liver mitochondria from chickens fed "Carbohydrate free diet". *J. Nutrition 99,* 109–112.

3. Amara, C. E., Shankland, E. G., Jubrias, S. A., Marcinek, D. J., Kushmerick, M. J., Conley, K. E. (2007) Mild mitochondrial uncoupling impacts cellular aging in human muscles in vivo. *Proc. Natl Acad. Sci. USA 104,* 1057–1062.
4. Bradford, M. H. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing principles of protein-dye binding. *Anal. Biochem. 72,* 248–252.
5. Brand, M. D., Pakay, J. L., Ocloo, A., Kokoszka, J., Wallace, D. C., Brookes, P. S., Cornwall, E. J. (2005) The basal proton conductance of mitochondria depends on adenine nucleotide translocase content. *Biochem. J. 392,* 353–362.
6. Brand, M. D., Chien, L., Ainscow, E. K., Rolfe, D. F. S., Porter, R. K. (1994) The causes and functions of mitochondrial proton leak. *Biochim. Biophys. Acta 1187,* 132–139.
7. Brand, M. D., Nicholls, D. G. (2011) Assessing mitochondrial dysfunction in cells. *Biochem. J. 435,* 297–312.
8. Brand, M. D., Turner, N., Ocloo, A., Else, P. L., Hulbert, A. J. (2003) Proton conductance and fatty acyl composition of liver mitochondria correlates with body mass in birds. *Biochem. J. 376,* 741–748.
9. Buchwalter, D. B., Cain, D. J., Martin, C.A., Xie, L., Luoma, S. N., Garland, T. (2008) Aquatic insect ecophysiological traits reveal phylogenetically based differences in dissolved cadmium susceptibility. *Proc. Natl Acad. Sci. USA 105,* 8321–8326.
10. Buttemer, W. A., Abele, D., Costantini, D. (2010) From bivalves to birds: oxidative stress and longevity. *Func. Ecol. 24,* 971–983.
11. Cawthorn, D. M., Steinman, H. A., Witthuhn, R. C. (2012) Evaluation of the 16S and 12S rRNA genes as universal markers for the identification of commercial fish species in South Africa. *Gene 491,* 40–48.
12. Duong, C. A., Sepulveda, C. A., Graham, J. B., Dickson, K. A. (2006) Mitochondrial proton leak rates in the slow, oxidative myotomal muscle and liver of the endothermic shortfin mako shark *(Isurus oxyrinchus)* and the ectothermic blue shark *(Prionace glauca)* and leopard shark *(Triakis semifasciata). J. Exp. Biol. 209,* 2678–2685.
13. Goglia, F., Silvestri, E., Lanni, A. (2002) Thyroid hormones and mitochondria. *Biosci. Rep. 22,* 17–32.
14. Guderley, H., Turner, N., Else, P. L., Hulbert, A. J. (2005) Why are some mitochondria more powerful than others: Insights form comparisons of muscle mitochondria from three terrestrial vertebrates. *Comp. Biochem. Physiol. 142B,* 172–180.
15. Hulbert, A. J., Else, P. L. (2004) Basal metabolic rate: History, composition, regulation, and usefulness. *Physiol. Biochem. Zool.* 77, 869–876.
16. Hulbert, A. J., Pamplona, R., Buffenstein, R., Buttemer, W. A. (2007) Life and death: metabolic rate, membrane composition, and life span of animals. *Physiol. Rev. 87,* 1175–1213.
17. Hulbert, A. J., Turner, N., Hinde, J., Else, P., Guderley, H. (2006) How might you compare mitochondria from different tissues and different species? *J. Comp. Physiol. 176B,* 93–105.
18. Kleiber, M. (1932) Body size and metabolism. *Hilgardia 6,* 315–353.
19. Lehninger, A. L., Nelson, D. L., Cox, M. M. (2008) *Lehninger Principles of Biochemistry* (5th edition). W. H. Freeman & Co., New York, USA, pp. 707–764.
20. Lemmon, A. R., Emme, S., Lemmon, E. M. (2012) Anchored hybrid enrichment for massively high-throughput phylogenomics. *Syst. Biol. 61,* 727–744.
21. Loeuille, N., Loreau, M. (2006) Evolution of body size in food webs: does the energetic equivalence rule hold? *Ecol. Lett. 9,* 171–178.
22. Motomura, H. (2004) Morphological comparison of a poorly known scorpion fish, *Parapterois macrura,* with a related species, *P. heterura* (Scorpaenidae: Pteroinae). *Zool. Stud. 43,* 1–7.
23. Nussbaum, R. A., Wu, S. (2007) Morphological assessments and phylogenetic relationships of the seychellean frogs of the family Sooglossidae (Amphibia: Anura). *Zool. Stud. 46,* 322–335.
24. Paital, B., Chainy, G. B. N. (2010) Antioxidant defenses and oxidative stress parameters in tissues of mud crab *(Scylla serrata)* with reference to changing salinity. *Comp. Biochem. Physiol. C 151*, 142–151.

25. Paital, B., Chainy, G. B. N. (2012) Effects of salinity on O_2 consumption, ROS generation and oxidative stress status of gill mitochondria of the mud crab *Scylla serrata*. *Comp. Biochem. Physiol. C 155,* 228–237.
26. Porter, R. K. (2001) Allometry of mammalian cellular oxygen consumption. *Cell. Mol. Life Sci. 58,* 815–822.
27. Rasmussen, H. N., Rasmussen, U. F. (1997) Small scale preparation of skeletal muscle mitochondria, criteria of integrity and assays with reference to tissue function. *Mol. Cell. Biochem. 174,* 55–66.
28. Rusyniak, D. E., Stephany, M. D., Tandy, L., Lisa, M. K., Jon, E. S., James, E. K. (2004) The effects of ecstasy (MDMA) on rat liver bioenergetics. *Acad. Emerg. Med. 11,* 723–729.
29. Subudhi, U., Das, K., Paital, B., Bhanja, S., Chainy, G. B. N. (2009) Supplementation of curcumin and vitamin E enhances oxidative stress, but restores hepatic histoarchitecture in hypothyroid rats. *Life Sci. 84,* 372–379.
30. Subudhi, U., Das, K., Paital, B., Bhanja, S., Chainy, G. B. N. (2008) Alleviation of enhanced oxidative stress and oxygen consumption of L-thyroxin induced hyperthyroid rat liver mitochondria by vitamin E and curcumin. *Chem. Biol. Interact. 173,* 105–114.
31. Theron, M., Guerrero, F., Sebert, P. (2000) Improvement in the efficiency of oxidative phosphorylation in the fresh water eel acclimated to 10.1 MPa hydrostatic pressure. *J. Exp. Biol. 203,* 3019–3023.
32. Turner, N., Hulbert, A. J., Else, P. L. (2006) Limits to physical performance and metabolism across species. *Curr. Opin. Clin. Metab. Care 9,* 691–696.
33. Veauvy, C. M., Wang, Y., Walsh, P. J., Perez-Pinzon, M. A. (2002) Comparison of the effects of ammonia on brain mitochondria function in rats and gulf toadfish. *Am. J. Physiol. Regul. Integr. Comp. Physiol. 283,* R598–R603.