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Establishment of dry-down hypoxic stress treatment protocol for snakehead freshwater fish, *Channa striatus*

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ABSTRACT
The air-breathing fish, *Channa striatus* can survive in muddy and marshy water. It is a hypoxia tolerant species. Hypoxia tolerance linked investigations for large-bodied fishes are limited in the absence of suitable laboratory-protocol. Here, a dry-down approach for hypoxia treatment in FRP (fiberglass reinforced plastics) tanks containing pond-mud and freshwater was investigated for 61 days. This facilitated lowering of dissolved oxygen (~0.15 mg/L from 39 days) in hypoxic tanks compared to ≥3.5 mg/L normoxic tanks. Hypoxia exposed fishes preferred benthic habitat with reduced locomotives and hibernated under soft-mud, whereas normoxic fishes remained active with normal air-gulping. Lactate dehydrogenase, an anaerobic glycolytic enzyme, levels were elevated in serum, liver, muscle and brain of hypoxic fishes. The heightened *hsp90β* gene, a stress marker, expression in hypoxic fishes was also documented. Such striking differences on the behavioral and metabolic levels were indicative of successful hypoxia stress treatment. Thus, a laboratory-based rearing protocol for investigating long-term hypoxia stress tolerance in *C. striatus* was established. This will also have future implications in extending this protocol in other fish species.

Keywords: *Channa striatus*; Hypoxia; Stress; Laboratory protocol.

1. Introduction
In aquatic habitats, fluctuations in water level leading to dry-down events often lead to the reductions in habitat area and dissolved oxygen (DO) levels. This eventually will have adverse effects on survivorship, growth and fertility. The gravity of negative impacts on fish community will depend on the degree of drying and adaptation capabilities (typically the physiological and behavioral adaptations) of an organism to compensate for environmental variability.

Channa striatus (Family: Channidae) is a tropical carnivorous freshwater fish[1-2]. It is regarded as a commercially important food fish with therapeutic value[1, 3-4]. *C. stratus* is an air-breathing fish that inhabits oxygen (O_2) deficient muddy and marshy waters[5] including hibernation by burrowing in soft mud or under hard-backed mud crust to survive temporary drought[5-7]. Thus, this species can survive in an environment where oxygen supply is limited. It is expected that behavioral and metabolic adaptations must be playing significant roles in order to withstand extreme hypoxic or anoxic conditions. *C. striatus* should be useful species for investigating the nature of tolerance with prolonged hypoxia. One of the major limitations for investigating hypoxia stress tolerance in this species has been lack of suitable laboratory-based protocol of stress treatment. In this study, we established a dry-down hypoxic stress treatment protocol for *C. striatus* for studying behavioral and physiological adaptations inside laboratory. This will have future implications in extending this protocol in other non-model fish species for studying detailed mechanistic pathways of adaptive tolerances against hypoxia stress.

2. Materials and methods
C. striatus, 12±0.27 cm length (~14 g) were collected from the Institute's farm. Fishes were reared in FRP (fiberglass reinforced plastics) tanks (L x B x H: 100 cm x 50 cm x 40 cm) containing 7.5 cm (H) mud and 80 liters pond water (freshwater) in triplicate during summer season, May and June, (local environmental temperature was 38±1 ºC, where recorded water temperature was ~32.4 ºC). Rearing tanks were placed under overhead shed exposing sunlight for six hours/day.

Ten individual fishes were reared in each tank Fishes were fed with poultry offal/trash fish in combination with rice bran @ 3% of body weight[3]. Hypoxic stress was imposed by allowing a gradual and progressive removal of water (one liter per day) in addition to natural evaporation from the tanks (dry-down) without any external aeration-support in the hypoxic tanks. This led to the reductions in habitat area and lowering of DO content as measured by ORION 3STAR Portable DO meter (Thermo Electron Corporation) over the period of times. Contrary to this, normoxic condition was maintained by exchanging water at regular intervals along with external aeration-support to maintain a constant DO level (≥3.5 mg/L).

Fishes were anaesthetized with MS-222 (Merck, Germany). Serum was prepared and organs like whole brain, portion of the liver and muscle were dissected. LDH activity in tissues was measured as described[8-10]. Briefly, excised fresh tissues were washed in ice-cold 0.9% NaCl, weighed and homogenized in 10 volumes of ice-cold homogenization solution (0.25 M sucrose, 20 mM imidazole, 20 mM NaF, 1 mM EDTA, 10 mM dithiothreitol and 0.5% Triton X-100; pH 7.2) containing few crystals of phenylmethylsulfonyl fluoride. Homogenates were spun at 12000 rpm for 30 min at 4 ºC. The reaction was performed with 20 µl supernatant, 1 mM pyruvate, 0.15 mM NADH and 1 mM EDTA at 25 ºC; and enzyme activity was assayed at 340 nm using UV-VIS spectrophotometer. Calculations were performed as described[10]. Serum LDH activity was measured using commercial kit (Crest Biosystems, India).

Quantitative real-time PCR (qPCR) for *hsp90β* and the reference genes were performed in triplicate for liver cDNA sample using Light Cycler-480 SYBR Green I kit (Roche Diagnostics, Germany) in a Light Cycler 480 RT-PCR instrument (Roche Diagnostics, Germany) as described[11-13]. The cDNA was generated from the pooled liver RNA samples (2 individuals of normoxic and hypoxic group independently X 4days such as 39, 45, 47 and 57 days of hypoxia treatments). Relative mRNA levels of target genes were normalized to *β-actin* expression for each sample, and normalized standard deviations were calculated. Primer used as fellows: *hsp90β* (sense 5'-TCATGAAGGCCCAGGCACT-3' and anti-sense 5'-TCTGTGGGTCATCCAGGGA-3') and *β-actin* (sense 5'-GTATGTGGCCATCCAGGCT-3' and anti-sense 5'-TAGCCACGCTCGGTCAGGAT-3'). Primer annealing temperature for target genes and *β-actin* was 58 °C. The possibility of genomic DNA contamination was ruled out by a negative control PCR containing RNA template and *β-actin* primers.

3. Results and discussion

C. striatus has been known to be capable of tolerating hypoxic stress due to water deficit or drought. In the absence of a suitable stress treatment protocol, it has been difficult to undertake extensive studies to find out some determinants of tolerance revealing its mechanisms. In this study, a laboratory-based protocol termed 'hypoxia stress treatment' imposing hypoxic environment in the rearing tanks. The dry-down protocol in pot experiments has been successfully employed to impose water stress in various plant systems[14]. Similar type of dry-down approach was undertaken in the mud containing water tanks to reduce DO levels gradually concomitant with the progressive loss of water quantities that facilitated hypoxic condition over the period of times. To get a profile that could reflect more closely to the real hypoxic environment, fishes were exposed (for a period of two months) to reduced-water containing muddy-water instead of maintaining constant level of freshwater. As shown in Fig. 1, the DO levels for normoxic tanks were steadily maintained at ≥3.5 mg/L throughout experiments, whereas it was lowered to 1 mg/L within 15 days for hypoxic counterpart. Further, DO levels were drastically reduced to 0.15 mg/L from 39 days onwards till 61 days in all the hypoxic tanks. These results indicated that fishes were exposed to hypoxic conditions for a long period.

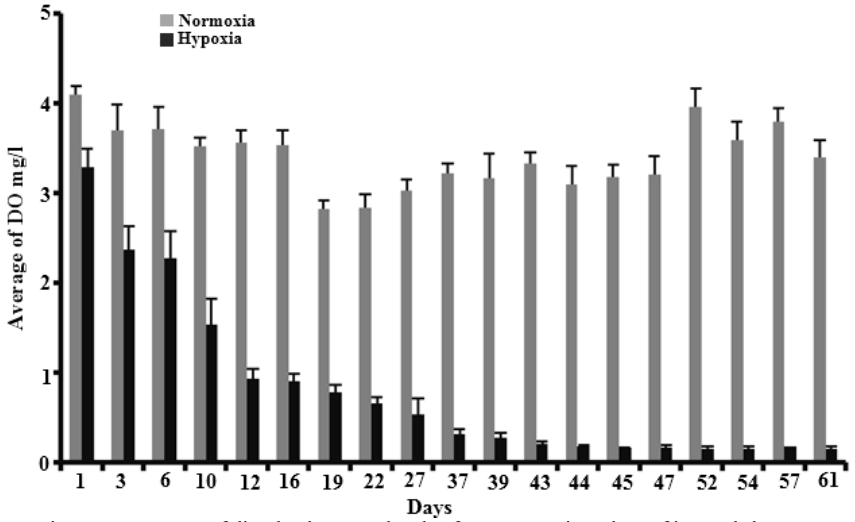

Fig 1: A representation of comparative measurements of dissolved oxygen levels of water at various days of intervals between normoxic and hypoxic tanks. The DO levels were dramatically reduced around 39 days onwards in hypoxic tanks. The data represent the average DO level.

Fishes in the hypoxic tanks preferred benthic habitat with inactivity and/or hibernated by burrowing in soft-mud, whereas fishes in normoxic tanks remained in all waters with normal activity of frequent air-gulping. Such behavioral differences, as observed in natural conditions, further confirmed that hypoxia stress treatment was successful.

Lactate dehydrogenase level has been considered as a hallmark marker for hypoxia stress linked to environmental habitat or injury/tumor[7, 15]. To confirm the efficiency of hypoxia stress treatment by dry-down approach, serum LDH level was measured at 39, 45, 47 and 57 days of hypoxia treatments since DO content remained far below (~0.15 mg/L)

than normal level (≥3.5 mg/L) in those days. As expected, the serum LDH content was increased by 1.5-fold in hypoxic fishes as compared to normoxic fishes (Fig. 2A). This was further heightened by 1.7- and 2.4-folds, respectively, on day 47 and 57 (Fig. 2A).

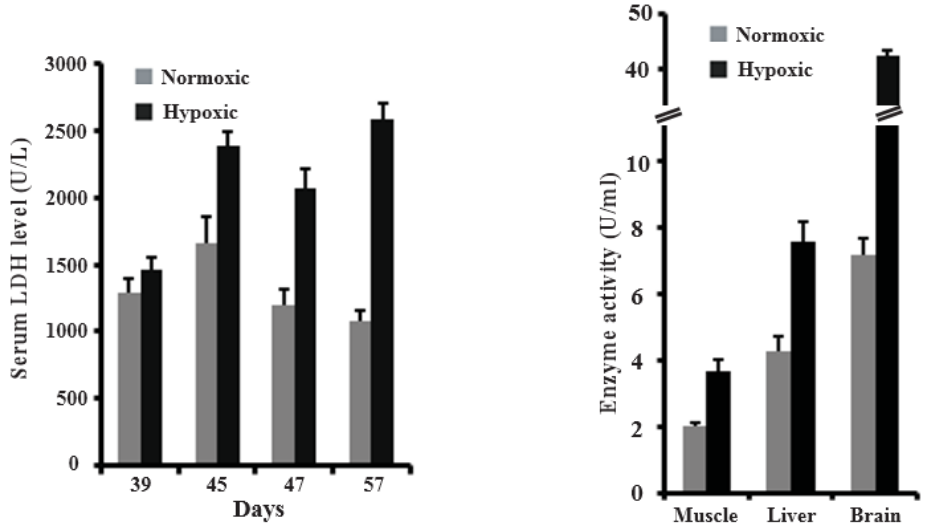

Fig 2: Effect of hypoxia stress treatment on glycolytic enzyme activities in *C. striatus*. (A) Comparative LDH levels in serum between normoxic and hypoxic fishes at various days (39, 45, 47 and 57). (B) LDH activity in liver, muscle, brain of normoxic and hypoxic fishes. The experiment was done in triplicate and the data shows the average of the triplicate experiments. LDH, lactate dehydrogenase; H, hypoxic; N, normoxic.

The elevated LDH clearly demonstrated that the fishes were under extreme hypoxic condition at least from 39 days to 61 days. The deficit of freshwater without aeration support reduced DO level as also habitat area enforcing fishes to remain in a dormant state as the means of behavioral and physiological adaptations to survive. The glycolytic pathway has been central to hypoxia tolerance in several fish species[7, 9, 16]. The altered activities of LDH, in white skeletal muscle, liver and brain of hypoxia treated *C. striatus* were analyzed. The LDH activity was detected to be heightened by 1.7-, 1.8- and 6-folds, respectively, in muscle, liver and brain in hypoxia than normoxia (Fig. 2B). This is in line with previous findings of Amazonian cichlids and sea scorpion under hypoxic stress[8, 16]. These findings suggested that anaerobic glycolytic pathway is in operational supporting the successful hypoxic stress treatment.

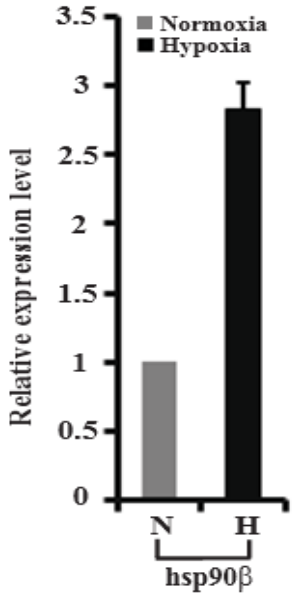

Fig 3: Expression pattern of *hsp90β* by qPCR in the liver of hypoxic and normoxic
C. striatus. The *hsp90β* gene was significantly up-regulated (~2.85-fold) in the liver of hypoxic fishes compared to normoxic fishes.

Heat shock proteins have long been believed as biomarkers for physiological stresses including hypoxic stress and *hsp90β* is a major regulator in HIF-1K activation[17-19]. In this study, the differential mRNA expression in the liver for the *hsp90β* isomer was analyzed between hypoxic and normoxic groups by using qPCR. The *β-actin* instead of Glyceraldehyde 3-phosphate dehydrogenase (*G3PDH)*, Cytochrome c oxidase subunit I (*CoI*) and Cytochrome c oxidase subunit II (*CoII)* was identified as the most stable reference gene by using geNorm method as described earlier[11-12]. As expected, the relative *hsp90β* gene expression was increased by ~2.85-fold in hypoxic liver than normoxic, further confirming successful hypoxic stress treatment (Fig. 3).

4. Conclusion

Our results demonstrated, for the first time, a successful establishment of laboratory-based rearing protocol for investigating long-term hypoxia stress tolerance in non-model large-bodied snakehead, *C. striatus*. There is a scope to extend this protocol in other fish species. It will certainly trigger future studies linked to the surveying of modulated gene expressions including physiological/biochemical adaptive mechanisms under short- and long-term hypoxia-stress in teleost.

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6. References

1. Baie SH, Sheikh KA. The wound healing properties of *Channa striatus* cetrimide cream- tensile strength measurement. J Ethnopharmacol 2000; 71(1-2):93-100.
2. Sahu B, Kumar K, Sahoo A, Mohanty U, Kumar R, Sahoo M *et al.* Processing and value addition to murrels in value chain. Fishing Chimes 2011; 31:106-8.
3. Kumar K, Eknath A, Sahu A, Mohanty U, Kumar R, Sahoo M *et al.* Snakeheads: Challenging fish for

diversification of fish farming. Fishing Chimes 2011; 31:110-3.
4. Sahu B, Kumar K, Sahoo ARK, Mohanty UL, Sahoo N *et al.* Carcass characteristics of marketable size striped murrel, *Channa striatus*. J Appl Ichthyol 2011; 28(2):258-60.
5. Gunther ACLG. An introduction to the study of fishes. New Delhi, India: Today and Tomorrow's Book Agency, 1980.
6. Chandra S, Banerjee T. Histopathological analysis of the respiratory organs of *Channa striatus* subjected to air exposure. Veterinarski Archiv 2004; 74:37-52.
7. Graham J. Air-Breathing Fishes: Evolution, Diversity and Adaptation. New York, USA, Academic Press, 1997.
8. Chippari-Gomes AR, Gomes LC, Lopes NP, Val AL, Almieda-Val VM. Metabolic adjustments in two Amazonian cichlids exposed to hypoxia and anoxia. Com Biochem Physiol 2005; 141(3):347-55.
9. Martinez ML, Landry C, Boehm R, Manning S, Cheek AO, Rees BB. Effects of long-term hypoxia on enzymes of carbohydrate metabolism in the Gulf killifish *Fundulus grandis*. J Exp Biol 2006; 209(19):3851-61.
10. Singh A. Enzyme Assays. New Delhi, India: Regency Publications, West Patel Nagar, 2007.
11. Barman HK, Patra SK, Das V, Mohapatra SD, Jayasankar P, Mohapatra C *et al.* Identification and characterization of differentially expressed transcripts in the gills of freshwater prawn (*Macrobrachium rosenbergii*) under salt stress. The Scientific World J 2012; 2012:149361.
12. Mohapatra C, Barman HK, Panda RP, Kumar S, Das V, Mohanta R *et al.* Cloning of cDNA and prediction of peptide structure of Plzf expressed in the spermatogonial cells of *Labeo rohita*. Mar Genomics 2010; 3(3-4):157-63.
13. Panda RP, Barman HK, Mohapatra C. Isolation of enriched carp spermatogonial stem cells from *Labeo rohita* testis for *in vitro* propagation. Theriogenol 2011; 76(2):241-51.
14. Deokar AA, Kondawar V, Jain PK, Karuppayil SM, Raju NL, Vadez V *et al.* Comparative analysis of expressed sequence tags (ESTs) between drought-tolerant and susceptible genotypes of chickpea under terminal drought stress. BMC Plant Biol 2011; 11:70.
15. Koudelova J, Rauchova H, Vokurkova M. Activity of lactate dehydrogenase in serum and cerebral cortex of immature and mature rats after hypobaric hypoxia. Neurochem Res 2006; 31(7):915-9.
16. Lushchak VI, Bahnjukova TV, Storey KB. Effect of hypoxia on the activity and binding of glycolytic and associated enzymes in sea scorpion tissues. Braz J Med Biol Res 1998; 31(8):1059-67.
17. Trisciuoglio D, Gabellini C, Desideri M, Ziparo E, Zupi G, Bufalo DD. Bcl-2 regulates HIF-1alpha protein stabilization in hypoxic melanoma cells via the molecular chaperone HSP90. PLoS One 2010; 5(7):e11772.
18. Xu Q, Liu Y. Gene expression profiles of the swimming crab *Portunus trituberculatus* exposed to salinity stress. Mar Biol 2011; 158:2161-72.
19. Minet E, Mottet D, Michel G, Roland I, Raes M, Remacle J *et al.* Hypoxia-induced activation of HIF-1: role of HIF-1alpha-Hsp90 interaction, FEBS Lett 1999; 460(2):251-6.