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EXPRESSION OF STRESS RESPONSE GENES IN MUD-DWELLING STINGING CATFISH *Heteropneustes fossilis* DURING EXPOSURE TO A DRYING MUD-BED

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ABSTRACT

The natural habitats of Indian catfish (*Heteropneustes fossilis*) are generally known for less oxygen and most often polluted water bodies. The present study was aimed to understand the adaptability in terms of antioxidant defense mechanism at transcript levels. The study considered key transcripts such as superoxide dismutase 2 (*sod2),* catalase *(cat),* glutathione peroxidase 4 *(gpx4),* and heat shock protein 70 *(hsp70),* heat shock cognate 70 *(hsc70),* hypoxia inducible factor 1alpha (*hif1a)* of the catfish exposed to a drying mud-bed while dwelling inside mud-peat under semidry conditions for 15, 30 and 45 days. The study revealed that desiccation stress triggered complicated oxidative stress. The *cat, sod2* and *gpx4* and *hsp70* mRNA expressions significantly up-graded in the mud-dwelled fish. Further, mud-dwelling also resulted in significant increase in the expression of *hif1α* in gills and muscle of fish after 30 days, suggesting its role as a vital regulator that carries out important alterations in gene expression under hypoxic stress. Collectively, the observations suggested that stress condition resulted in the development of oxidative stress, and *H. fossilis* was able to respond through alterations in stress responsive genes to survive semi-dry conditions.

Keywords: Desiccation, hypoxia, *hif1α*, reactive oxygen species, antioxidant defense mechanism

INTRODUCTION

The aquatic fauna suffers from a number of environmental stressors and one such stress is the drying of water bodies. Such physical disturbance may occur naturally and also due to anthropogenic activities. The drying of water bodies due to seasonal or at any other occasions due to varied climatic conditions leads to desiccation resulting in hypoxia. Hypoxia can be described as a condition under which the dissolved oxygen levels decrease below the normal. Oxygen plays an important role in the physiology of fishes (Hughes, 1973). Surprisingly, some aquatic organisms have adaptation or physical tolerance against such disturbances. In addition, the low oxygen availability during hypoxia in fish results in the disruption of energy balance as most of the oxygen consumed is utilized for the production of ATP (Richards, 2009).

Hypoxia may cause oxidative stress leading to the generation of reactive oxygen species (ROS) (Duranteau *et al.*, 1998). These are the reactive molecules that result from molecular oxygen and include hydrogen peroxide and free radicals (superoxide radical, hydroxyl radical, peroxyl radical, etc.). Overproduction of these molecules leads to deleterious changes and destruction of cellular

structures causing oxidative stress. However, to combat this oxidative stress the antioxidant defense system works to regulate the concentrations of these reactive molecules (Baker *et al*., 2007). In defense system, superoxide dismutase and catalase enzymes are the primary antioxidant enzymes and considered the first line of defense against oxidative stress (Pandey *et al*., 2003). These enzymes are commonly used as bio-markers to assess the production of ROS (Monteiro *et al*., 2006). Several other markers can also be monitored during stress conditions, which include heat shock proteins (hsps) which are a family of proteins that help in cell function during normal and stress conditions (Parsell and Lindquist, 1993). This family of proteins includes *hsp70* whose expression is induced in response to stressors. However, other hsps like heat shock cognate70 (*hsp70*) is expressed constitutively in unstressed conditions. These proteins play important role as molecular chaperones that are vital for functioning of proteins (Feder and Hofman, 1999). Hypoxia also involves the expression of certain transcription factors. One such transcription factor is hypoxia-inducible factor (HIF), which is a heterodimer. This is an important transcription factor that regulates the expression of genes during hypoxic conditions (Guan *et al*., 2014). Several studies suggest that there are three isoforms of *hif* in vertebrates which include *hif-1α*, *hif-2α* and *hif-3α*. Each of these regulates different functions and among these the acute hypoxic response is mainly regulated by *hif-1α* (Nambu *et al*., 1996; RytKönen *et al*., 2011; RytKönen *et al*., 2013).

Freshwater catfish, *Heteropneustes fossilis*, is an air-breathing fish which belongs to Heteropneustidae family and possesses an elongated body with characteristic dorsoventrally flattened head (Biswas and Kaviraj, 2002). It is mostly found in muddy and marshy water bodies with low level dissolved oxygen levels. *H. fossilis* is mainly distributed throughout Indian sub-continent (Ahmed *et al*., 2013). The air-breathing catfish can rely on aerial respiration during low-oxygen availability in the surrounding water. This capacity of fish is aided by air-sacs present within the body (Thakur, 1991) and is more resistant to various environmental constraints like ammonia, hypoxia, desiccation stresses, etc. (Saha and Ratha, 1998; Saha and Ratha, 2007). It naturally goes inside the mud during dry season and survives well inside. Hence, the present study was designed to understand the impact of desiccation on this species. The study was aimed to know the impacts of desiccation and low dissolved oxygen on the adaptability of fish and also help to understand how it is regulated through transcript expression patterns of the various stress responsive genes.

MATERIALS AND METHODS

Experimental animals

The stinging catfish (*Heteropneustes fossilis*) of 15 ± 10 g body weight for the present study were purchased from a local fish farm. Fishes were acclimatized in laboratory for one month at 28 ± 2°C under 12/12 h light and dark photoperiod condition. Fishes were fed with a formulation containing rice bran and dried fish powder (5% of body weight) every day (Choudhury and Saha, 2012). On every alternate day the water was changed. No sex differentiation of fish was done during these studies. Food was withdrawn 24 h before the experiments.

Experimental set up

An artificial mud peat was constructed using a cemented tank. The tank was filled with mud upto 45 cm height and a set of 30 acclimatized catfishes were released into the mud-peat for experimental period of 15, 30 and 45 days. The mud-peat was maintained by regular sprinkling of water over the mud to avoid complete drying of mud (Choudhury and Saha, 2012). A different set of 10 fishes served as control group and was maintained under laboratory conditions. The dissolved oxygen (DO) level of water in both control and mud-peat conditions were measured after 15, 30 and 45 days using a DO meter 314 (Systronics). The DO level of water under controlled condition was 7.5, 7.2 and 7.6 ppm whereas that of mud-peat water was 6.4, 3.6 and 2.8 ppm after 15, 30 and 45 days, respectively. No

fish mortality was observed during the experimental period. After 15, 30 and 45 days, three fishes each from control and mud-peat were removed and anesthetized in neutralized 3-aminobenzoic acid ethyl ester for 5 min. Tissues from liver, kidney, gills and muscle were dissected out, dipped in liquid nitrogen and stored at -20°C.

RNA extraction and cDNA synthesis

The total RNA was extracted from the tissues of *H. fossilis* using RNAiso Plus (Takara, Japan) following the method of Rio *et al*. (2010). Isolated total RNA was quantified in a Qubit® 3.0 fluorometer (Thermo Fisher Scientific) and further integrity confirmed by electrophoresis on 1% agarose gel. First strand cDNA was synthesized from 500 ng of total RNA in a total volume of 10 µL with iScript™ cDNA Synthesis kit (Bio-Rad) following the manufacturer's protocol. For qPCR studies, the cDNA samples were diluted 50 times with sterile miliQ water.

Designing of primers

For qPCR amplification, the gene specific primers for *sod2, cat, gpx4, hsp70, hsc70* and *hifla* were designed using *sod2, cat, gpx4, hsp70, hsc70* and *hifla* species-specific cDNA sequences available in the NCBI database (accession numbers given below) with the help of primer-BLAST (NCBI) and the properties were checked using OligoCalculator. The size of amplicons was approximately 160 bp. The specificity of primers was checked by inspecting the PCR amplicons on 1.2% agarose gel. The details of primer pairs for *H. fossilis* are as under:

Gene names	Abbre-viation	Primer sequences (5'⟶ 3')	Melting temperature (°C)	Annealing temperature (°C)	GenBank accession number
Superoxide dismutase 2	*sod2*	F = GAGACCTCACACAGGACTGC R = CCCAGATCACCAACATGCCT	F = 57.5 R = 57.5	51	MH938563
Catalase	*Cat*	F = CCGACACAATGACCACAGGA R = AAAGTGGGCCATCTCGTCAG	F = 57.2 R = 57.4	49	MH938564
Heat shock protein 70	*hsp70*	F = GAATTCATCAGCCTCCGCCA R = CAAGATCGCATCCAAGCTGC	F = 57.7 R = 56.9	49	MH938565
Heat shock cognate 70	*hsc70*	F = CTTGTCCTCCAGCACTCAGG R = GGTTCCACGGAAGAGGTCAG	F = 57.6 R = 57.5	48	MH938566
Glutathione peroxidase 4	*gpx4*	F = TTGGGTCATCCATTGGTCCG R = GAGACAGCGCTCATCCTCTC	F = 57.5 R = 57.3	48	MH938567
Hypoxia inducible factor 1 alpha	*hif1a*	F = AACAGACCGTGCCTGAGAAG R = CACAGAGCATGACAGGTGGT	F = 57.2 R = 57.3	49	MH938568
Beta actin	*β-actin*	F = CAGCTGAGCGTGAAATCGTG R = TCCAGAGAGGATGAGGAGGC	F = 62.5 R = 62.5	50	FJ409641

qPCR analysis

To determine the expression pattern and quantify *sod2, cat, gpx4, hsp70, hsc70* and *hif1a* mRNA in respective tissues of *H. fossilis* exposed to desiccation stress, relative quantitative RT-PCR was performed on cDNA produced from total RNA. The relative abundance of mRNA transcripts, in each sample, was normalized to the amount of an endogenous gene *H. fossilis β-actin.* qPCR was performed with Rotor Gene Q SYBR Green PCR (Qiagen). In a 25 µL reaction volume, 12.5 µL QuantiNova™ 2x Rotor-Gene SYBR Green PCR Master mix (Qiagen), 1.2 µL of each forward and reverse primers (10 µM) and 5 µL of cDNA template was used; and qPCR reactions were performed with thermal cycling conditions as: denaturation at 95°C for10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, primer annealing at respective annealing temperatures for 30 sec and extension at 72°C for 30 sec. The relative expressions of gene transcripts in treated groups were expressed as the fold change to the control by using the standard $2^{-\Delta\Delta Ct}$ method (Livakand Schmittgen, 2001; Kubista *et al*., 2006). The melt curve analysis was performed to check for any non-specific amplification during qPCR analysis (Supplementary data is available on *www.indianjournals*.com website in this particular article).

Chemicals

RNAiso Plus (Takara, Japan), Qubit Reagents (Thermo Fisher Scientific), iScript™ cDNA Synthesis kit (Bio-Rad), QuantiNova™ 2x Rotor-Gene SYBR Green PCR Master mix (Qiagen).

Statistical analysis

Experiments were conducted in triplicates and all the parameters were presented as mean ± SEM. The statistical significance was worked out by one-way ANOVA at 5% significance level, followed by Tukey's post-hoc test using SPSS software.

RESULTS AND DISCUSSION

The expression of different mRNAs was performed by qPCR analysis with the total RNA extracted from the tissues of mud-dwelled fish after 15, 30 and 45 days in parallel with control tissue. There was a uniform expression of *β-actin* in liver, kidney, gills and muscle of both control and mud-dwelled fish. The changes in the expression patterns of the stress response genes in stinging catfish *H. fossilis* on exposure to drying mud bed were demonstrated in the present study. The results clearly indicated that this air-breathing catfish could survive the desiccation stress through mechanisms that involve alterations in the expression pattern of different stress responsive genes. It is evident from different studies that during hypoxic condition there is an increase in the level of ROS (Mustafa *et al*., 2012). These reactive molecules are produced as a result of incomplete reduction of molecular oxygen during respiration (Staples and Buck, 2009). There are several enzymes that constitute the antioxidant defense system which play important role in detoxifying the ROS **(**Baker *et al*., 2007). Some of the important antioxidant enzymes mostly studied as bio-marker of oxidative stress include catalase, superoxide dismutase, glutathione peroxidase, etc. In this study, the expression of genes that encode these enzymes has been studied to evaluate the oxidative responses of *H. fossilis* under the given stress condition. Their expression significantly increased in different tissues under different experimental periods of stress. It can be speculated that desiccation stress may cause an oxidative response in *H. fossilis.* The alterations in the expression patterns of these genes altering the activities of antioxidant enzymes will help to get rid of the oxidative stress.

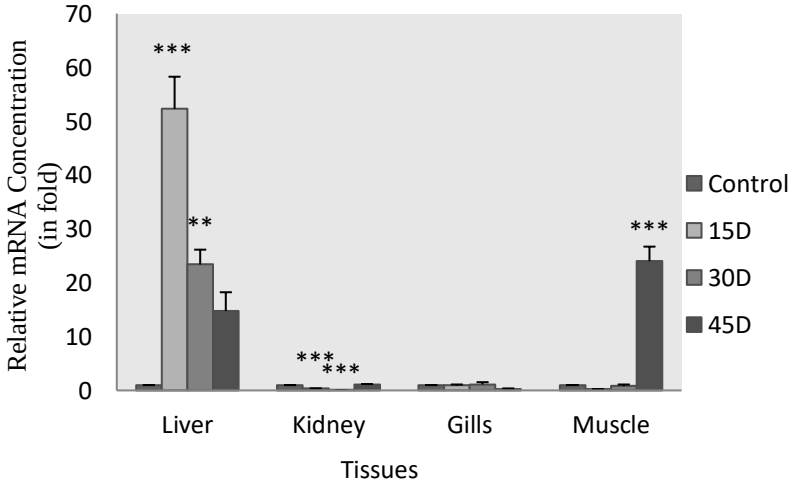

Fig. 1: R**elative mRNA expression levels of *cat* in liver, kidney, gills and muscle of *H. fossilis* at 15, 30 and 45 days of desiccation stress.** Levels of *cat* transcripts in exposed groups are expressed as fold changes relative to the control group after being normalized against *β-actin* standard. Data shown as mean ± SE (n = 3) of relative concentrations ($2^{-\Delta\Delta Ct}$ method, Livak *et al.,* 2001). The values are statistically significant at *p<0.05; **p<0.01; ***p<0.001 as compared to the control group (one-way ANOVA).

The *cat*, *sod2* and *gpx4* mRNA expression in liver, kidney, gills and muscles of mud-dwelled fish was up-regulated. *cat* expression significantly increased by 51.3-fold ($p<0.001$) and 22.4-fold ($p<0.01$) in the liver of fish exposed to 15 and 30 days of desiccation respectively as compared to the control group. In muscle a significant increase of 23-fold was observed in the

group exposed to 45 days of desiccation as compared to the control group. However, a significant decrease of 0.65-fold ($p<0.001$) and 0.95-fold ($p<0.001$) was observed in its expression in kidney in the group exposed for 15 and 30 days (Fig. 1). Catalase is mainly responsible for the regulation of the concentration of hydrogen peroxide (H_2O_2). H_2O_2 is also produced during hypoxic condition (Trasvina-Arenas *et al*., 2013). This enzyme catalyzes decomposition of H_2O_2 to water and oxygen. Expression of *sod2* was significantly increased by 7.97-fold ($p<0.05$) in liver in the group exposed for 30 days. A significant increase of 63- and 86-fold ($p<0.001$) was observed in the kidney of the group exposed to 15 and 30 days of desiccation, respectively, as compared to the control group. Similarly, it enhanced significantly by 27-, 79.3- and 55.8-fold ($p<0.001$) in gills of fish exposed for 15, 30 and 45 days, respectively, as compared to the control. *sod2* expression also increased significantly by 1.1-fold ($p<0.01$) in the muscle of fish exposed for 30 days as compared to the control (Fig. 2). The *sod2* codes for an enzyme that detoxifies superoxide radicals in the mitochondria during oxidative phosphorylation. *sod2* is the mitochondrial enzyme that exist as a homo-tetramer. Limited supply of oxygen during respiration may result in the excessive production of ROS that may attack the mitochondria itself and thus low ATP production (Paradies *et al*., 2002). The gills are chief respiratory organs of air-breathing catfish and hence suffer significantly from oxidative stress (Tripathi *et al*., 2013).

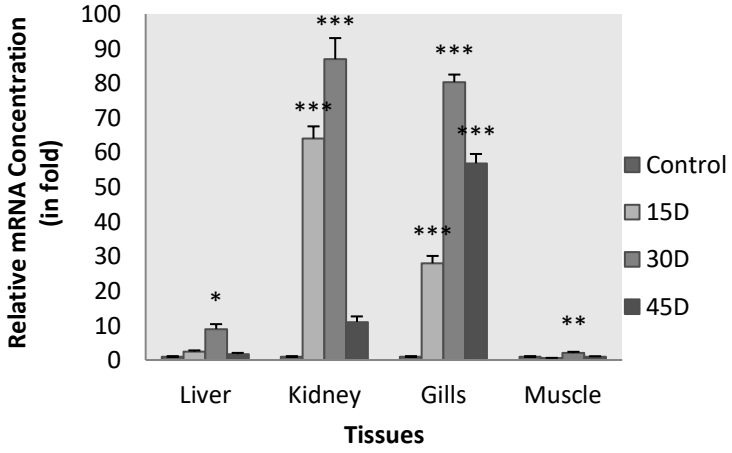

Fig. 2: Relative mRNA expression levels of *sod2* in liver, kidney, gills and muscle of *H. fossilis* at 15, 30 and 45 days of desiccation stress. Levels of *sod2* transcripts in exposed groups are expressed as fold changes relative to the control group after being normalized against *β-actin* standard. Data shown as mean ± SE (n=3) of relative concentrations ($2^{-\Delta\Delta Ct}$ method, Livak *et al*., 2001). The values are statistically significant at *p<0.05; **p<0.01; ***p<0.001 as compared to the control group (one-way ANOVA).

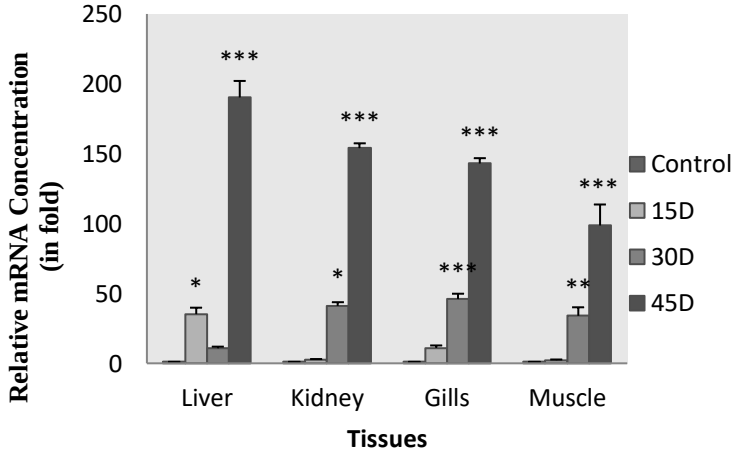

Fig. 3: Relative mRNA expression levels of *gpx4* in liver, kidney, gills and muscle of *H. fossilis* at 15, 30 and 45 days of desiccation stress. Levels of *gpx4* transcripts in exposed groups are expressed as fold-changes relative to control group after being normalized against *β-actin* standard. Data shown as mean ± SE (n = 3) of relative concentrations ($2^{-\Delta\Delta Ct}$ method, Livak*et al.*, 2001). The values are statistically significant at *p<0.05; **p<0.01; ***p<0.001 as compared to the control group (one-way ANOVA).

Significant increase in *gpx4* expression with 37.2-fold ($p<0.05$) in liver exposed for 15 days, 40.12-fold ($p<0.05$) in kidney, 45.0-fold ($p<0.001$) in gills and 33.0-fold ($p<0.01$) in muscle exposed to 30 days as compared to the control group. Its expression significantly increased by 189.3-, 153.0-, 142.0- and 97.65-fold in liver,

kidney, gills and muscles, respectively, in the group exposed for 45 days (Fig. 3). In mitochondria, in absence of catalase the important antioxidant against superoxide anion and H_2O_2 are sod as well as gpx. However, during oxidative stress the scavenging of H_2O_2 in cytosol is mostly performed by catalase (Salvi *et al*., 2007). This indicates that the fish could survive the oxidative stress during hypoxic condition as glutathione peroxidase4 is an antioxidant enzyme that prevents cells from lipid peroxidation. Lipid peroxidation occurs when oxygen reacts with lipids, thus forming various reactive molecules. This enzyme is important as it reduces H_2O_2 and lipid peroxides produced during oxidative stress and thus protecting the cell (Liang *et al*., 2007).

The present study showed that the DO level of the mud-peat water during 15th day was 6.4 ppm, which is quite normal for the fishes (Svobodova *et al*., 1993). Nevertheless, the study shows that the fishes suffered oxidative stress during 15 days of mud-peat exposure as evident from the expression levels of antioxidant enzyme transcripts. This oxidative stress can be attributed to the sudden restriction in feeding, which might have led to the oxidative stress (Pascual *et al*., 2002). Deprivation or reduction in feeding results in the production of more reactive oxygen molecules mainly in liver (Robinson *et al*., 1997). However, the fish could readapt to the mud-peat conditions and the oxidative stress experienced during the later experimental period can be attributed to the hypoxic conditions.

We also investigated the *hsp70* and *hsc70* expression levels in response to desiccation in liver, kidney, gills and muscle. No significant change in *hsc70* expression was observed as compared to the control. However, a significant decrease of 0.73-fold ($p<0.001$) in *hsc70* expression was observed in the kidney of fish exposed to 15 days of desiccation (Fig. 4). In contrast, the expression level of *hsp70* significantly increased by 2.64-fold ($p<0.01$) in liver and 2.61-fold ($p<0.01$) in gills of fish exposed to desiccation for 45 and 30 days, respectively, as compared to the control. However, *hsp70* expression was significantly down-regulated by 0.88-fold ($p<0.001$) and0.81-fold ($p<0.001$) in the muscle of fish exposed to desiccation for 15 and 30 days as compared to the control (Fig. 5). qPCR analysis showed up-regulation of *hsp70* expression in liver, gills and muscles, indicating that *hsp70* responses are sensitive to low DO exposure. Environmental stress in fishes can be studied using *hsps* as biomarkers (Iwama *et al*., 1998). It has been reported that during hypoxia-like stressors there is increase in the expression of *hsps* (Gornati *et al*., 2004; Wang *et al*., 2007). Moreover, the increased expression of *hsp70* might be a strategy adopted by *H. fossilis* to survive the oxidative stress-induced cellular destruction during hypoxia. Hsps play vital role in alleviating the protein destruction that might occur during the oxidative stress (Kalmar and Greensmith, 2009). While dwelling inside the mud-peat, the fishes get exposed to ammonia stress that may lead to accumulation of ammonia in various tissues (Chew and Ip, 2014). When exposed to air, *H. fossilis* may suffer oxidative stress as a consequence of ammonia stress (Paital, 2013). Further, when exposed to air there

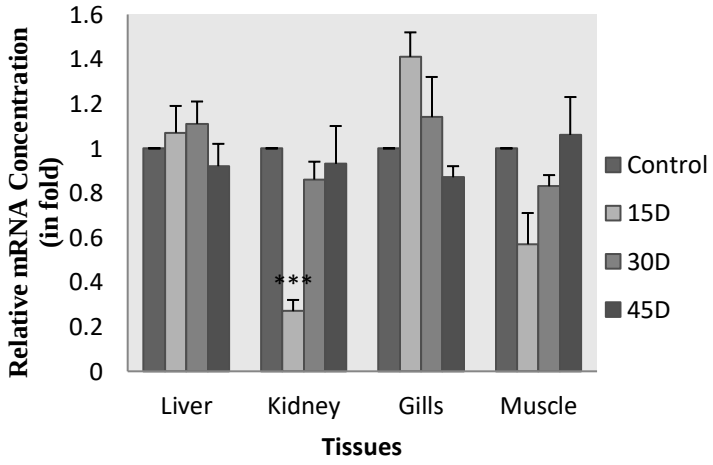

Fig. 4: Relative mRNA expression levels of *hsc70* in liver, kidney, gills and muscle of *H. fossilis* at 15, 30 and 45 days of desiccation stress. Levels of *hsc70* transcripts in exposed groups are expressed as fold changes relative to the control group after being normalized against *β-actin* standard. Data shown as mean ± SE (n = 3) of relative concentrations ($2^{-\Delta\Delta Ct}$ method, Livak *et al.,* 2001). The values are statistically significant at *$p<0.05$; **$p<0.01$; ***$p<0.001$ as compared to the control group (one-way ANOVA).

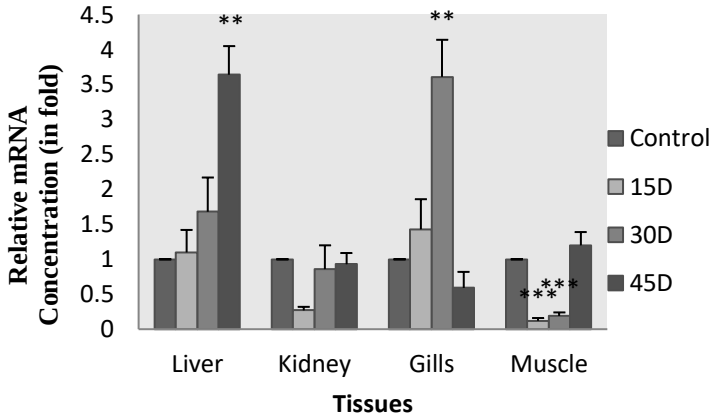

Fig. 5: Relative mRNA expression levels of *hsp70* in liver, kidney, gills and muscle of *H. fossilis* at 15, 30 and 45 days of desiccation stress. Levels of *hsp70* transcripts in exposed groups are expressed as fold changes relative to the control group after being normalized against *β-actin* standard. Data shown as mean ± SE (n = 3) of relative concentrations ($2^{-\Delta\Delta Ct}$ method, Livak *et al.*, 2001). The values are statistically significant at *p<0.05; **p<0.01; ***p<0.001 as compared to the control group (one-way ANOVA).

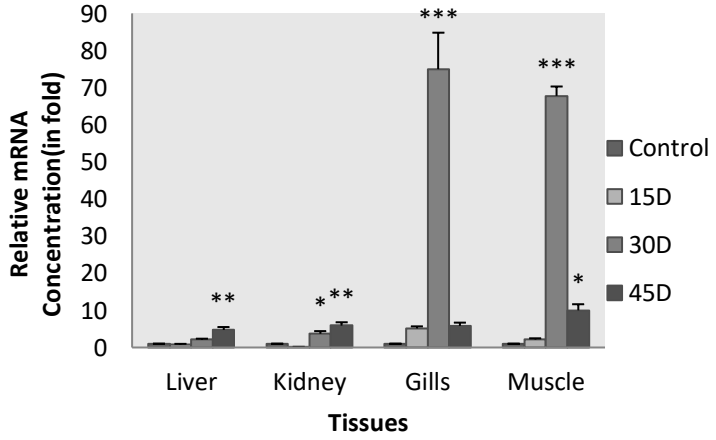

Fig. 6: Relative mRNA expression levels of *hif1α* in liver, kidney, gills and muscle of *H. fossilis* at 15, 30 and 45 days of desiccation stress. Levels of *hif1α* transcripts in exposed groups are expressed as fold changes relative to the control group after being normalized against *β-actin* standard. Data shown as mean ± SE (n = 3) of relative concentrations ($2^{-\Delta\Delta Ct}$ method, Livak *et al.*, 2001). The values are statistically significant at *p<0.05; **p<0.01; ***p<0.001 as compared to the control group (one-way ANOVA).

could be an increased buildup of ammonia in various tissues of *H. fossilis* as reported by Ratha *et al.* (1995).

The air-breathing catfish might survive this stress through increased expression of *hsp70* proteins and these proteins might prevent the denaturation and misfolding of cellular proteins. Surprisingly, no significant increase or decrease was observed in the levels of *hsc70* mRNA transcripts in various tissues as compared to the controls in the study. *hsc70* is normally expressed in all tissues during unstressed conditions and thus maintains the normal cellular homeostasis. It has been reported that ammonia stress in mud eel causes induction of *hsp70* gene and no significant changes in the expression of *hsc70* (Hangzo *et al*., 2017).

In addition, the expression of *hif1α* mRNA in liver, kidney, gills and muscle of mud-dwelled fish was significantly up-regulated. Its expression was significantly increased by 2.71-fold (p<0.05) in kidney, 74-fold (p<0.001) in gills and 66.78-fold (p<0.001) in the muscle of fish exposed to the desiccation for 30 days. The fish exposed to desiccation stress for 45 days also showed increased expression of *hif1α* by 3.72-fold (p<0.01) in liver, 4.91-fold (p<0.01) in kidney and 9.02-fold (p<0.05) in muscle (Fig. 6). The up-regulation of *hif1α* may be attributed to low oxygenation in these tissues. HIF is a heterodimer consisting HIF1α and HIF1β. Among the two, β subunit is similar to aryl hydrocarbon nuclear translocator (ARNT) and α-subunit is exclusive for HIF-1 (Rissanen *et al*., 2006). The HIF1α subunit stability is mainly responsible for the regulation of HIF function for oxygen sensitivity (Huang *et al.*, 1996).

During hypoxia, transcription factors play a vital role in the induction of transcription of several genes that are engaged in glycolysis, vasodilation, cell proliferation, transport of glucose, erythro-

poiesis as well as angiogenesis that may increase abruptly and thus curtail the damaging effects of oxygen deprivation at cellular levels. This increase of hypoxia-induced gene expression is promoted by HIF-1(Semenza, 1998; Wenger, 2002). All these events may help in promoting blood supply and oxygenation of the tissues. In hypoxic condition, the α subunit translocates into the nucleus and binds with ARNT forming the dimer, which then binds to the hypoxia-responsive elements (HRE) either in promoter or enhancer region of the corresponding genes (Lando *et al*., 2002). Therefore, it is necessary to determine whether the expression of *hif1α* would serve as a regulator in different physiological adjustments against the desiccation stress and is important for enhancement in adaptability and survivability of this air-breathing stinging catfish in such condition.

Conclusion: The results revealed that *H. fossilis*, an air-breathing catfish, on exposure to different periods of desiccation stress resulted in the alteration of different stress response gene expression. These physiological alterations might be correlated with its capacity to tolerate the hypoxic stress in the drying mud-bed condition.

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Ethical statement: The study was approved by the Institutional Animal Ethics Committee (IAEC) of Gauhati University, Guwahati, India. All methods involving animals were carried out in accordance with relevant guidelines and regulations.

Conflict of interest: The authors declare no conflict of interest.

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