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RESEARCH PAPER

Hypoxia-Induced Gene Expression Profiling in The Liver of Freshwater Fish, *Channa striatus*

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Abstract

Channa striatus is a large-bodied fish of freshwater habitat capable of withstanding hypoxic conditions. Adaptive response to hypoxia is a complex physiological process. A laboratory-based rearing protocol for investigating long-term hypoxia stress tolerance in this species was established. Using suppression subtractive hybridization technique, we examined gene expression patterns in liver during prolonged hypoxia exposure. A total of 130 transcripts from the enriched cDNA library, under hypoxic condition, were sequenced. BLAST analysis identified 58%, 18% and 24% as known, uncharacterized and unknown sequences, respectively. All known genes represented a broad spectrum of biological pathways such as transcription/translation, signal transduction, electron transport, immune response, reproduction, cellular transportation. Heightened abundances for 11 Known, 1 uncharacterized and 1 unknown mRNA in the hypoxic liver were documented. Among these, the full-length cDNA sequences for heat shock protein 90β and CSHL-338 clone (uncharacterized) were generated using RACE strategy. Full-length cDNA sequences of prefoldin and fatty acid binding protein was obtained from the respective clones of SSH cDNA library, were also up-regulated during hypoxia stress. In this study, possible physiological significances about hypoxia-tolerance transcripts have been discussed. The ESTs presented here will have potential future implications in exploring new mechanisms of hypoxia acclimation and/or tolerance in *C. striatus*.

Keywords: Hypoxia, *Channa striatus*, SSH, gene expression, gene ontology.

Introduction

Limitation of oxygen availability (hypoxia) in water bodies is a common phenomenon that imposes stress to aquatic organisms. Tolerance of aquatic animals to such a hypoxic stress is believed to be associated with dormancy with regard to inactivity and hypo-metabolism (Storey, 2007; Richards, farrell, & Brauner, 2009; Crans, Pranckevicius, & Scott, 2015). Hypoxia stress affects the growth and development of commercially important plants and animals including fishes. Development of stress-tolerant species is an urgent task in plant/fish breeding. Investigations on behavioral and physiological adaptive mechanisms, being operated in fish species under hypoxia stress, will have a positive impact not only on basic understanding of novel pathways, but also tolerance improvements for the commercially important fish species.

The cDNA library generated by suppression subtractive hybridization technique has been resourceful, particularly in the absence of prior genetic knowledge, in identifying organ-specific ESTs including stress-tolerant ESTs in several species of plants and aquatic species (Gracey, Troll, & Somero, 2001; Fu *et al*., 2005; Barman *et al*., 2012; Fan *et al*., 2014; Goswami *et al*., 2016). Recent advancement of transcriptome analysis by Next Generation Sequencing generates a resourceful huge data set, but difficult to assemble those data, especially in the absence of reference sequence; whereas SSH could efficiently identify a limited but definite ESTs. Hypoxia-induced gene expression profiling has also been studied in several fish species using cDNA microarrays revealing tissue-specific patterns of expression (Gracey *et al*., 2001; Ton, Stamatiou, & Liew, 2003; Brouwer, Brown-Peterson, Hoexum-Brouwer, Manning, & Denslow, 2008; Martinovic *et al*., 2009; Leveelahti, Leskinen, Leder, Waser, & Nikinmaa, 2011). Microarrays are based on the availability of abundant genes or gene segments with known sequences, whereas SSH technique can identify unknown sequences and appears to produce less false positive sequences as compared to other methods (Diatchenko, Lukyanov, Lau, & Siebert, 1999). Previously, the assessments of gene expression

modulations, in response to hypoxia to the blue crab (*Callinectes sapidus*), were performed with the help of microarray and SSH techniques (Brown-Peterson *et al*., 2005). The response of the Pacific oyster (*Crassostrea gigas*) to hypoxia under experimental conditions was focussed on the analysis of the differential expression patterns of specific set of genes (David, Tanguy, Pichavant, & Moraga, 2005). Hence, SSH technique has proven its usefulness to investigate gene expression (mRNA) patterns during hypoxia tolerance.

Channa striatus (Family: Channidae) has known to be a hypoxia tolerant freshwater species of Asian and African countries (Gunther, 1880; Graham, 1997). Being a large-bodied fish species, it was essential to undertake long-term investigations linked to their physiological tolerances against dramatically depleted O_2 content in the water bodies, mimicking natural stress conditions. Several studies linked to the impact of hypoxia stress, in large-bodied fish species such as rainbow trout (*Oncorhynchus mykiss*), were undertaken by sort exposure to hypoxic conditions (Bernier, Harris, Lessard, & Randall, 1996; Gamperl, Faust, Dougher, & Rodnick, 2004; Overgaard *et al*., 2004). Recently, we established a laboratory-based hypoxia-stress-treatment protocol of the prolonged period in *C. striatus* (Mohapatra, Kumar, Jayasankar, & Barman, 2013). Such experiment was in line with the fact that *C. striatus* is an air breathing fish that inhabits oxygen (O_2) deficient muddy and marshy water, including the hibernation by burrowing in soft mud or under hard mud crust, to survive temporary drought (Gunther, 1880; Graham, 1997; Chandra & Banerjee, 2004). This has provided an avenue to undertake laboratory-based investigations linked to behavioral and physiological adaptations against prolonged hypoxia-stress.

Liver is known to be among the most critical for facilitating hypoxia adaptation in fish species (Fraser *et al*., 2006; Flight, Nacci, Champlin, Whitehead, & Rand, 2011). Here, we exploited SSH mediated cDNA library construction from the liver of *C. striatus*, exposed to hypoxic condition for a longer period. ESTs were analyzed and compared with the known genes available in the database. The results were used to assign putative functions for known cDNAs. The gene ontology (GO) annotation, analysis provides an opportunity to predict the functions of gene sequences. Few known and uncharacterized/ unknown categories were identified as the possible hypoxia tolerant ESTs from the differential expression analysis. The full-length cDNA sequence information was also generated for selected transcripts. Relevance of the data with respect to likely hypoxia stress tolerance is discussed. Our study provides the basis of modulated gene expression (mRNA) patterns in response to hypoxia-stress in such an important non-model fish species, *C. striatus*.

Materials and Methods

Hypoxia Treatment

Dry down hypoxia stress treatments, mediated by gradual and progressive rearing water deficits, were given to *C. striatus* 12±0.27cm (~14g) fingerlings for 61days as described (Mohapatra *et al*., 2013). Dry-down approach was undertaken in the mud containing water tanks, by reducing O_2 levels gradually concomitant with the progressive loss of water quantities, thus facilitating hypoxic condition for 61 days. DO (dissolved oxygen) levels were drastically reduced to 0.15 mg/L (measured by DO meter, Thermo electron corporation) from 39 days onwards till 61 days in all the hypoxic tanks, whereas its steadily maintained (at ≥3.5 mg/L DO) throughout the experiments in normoxic tanks. Importantly, the water deficiency (and so lowering O_2 content) led to the typical behavioral changes such that of less physical activities and hibernation by burrowing in soft mud (also known as estivation), instead of frequent air breathing as seen in normoxic fishes.

RNA Extraction and Construction of Subtracted cDNA Library

Total RNA was extracted from the liver of *C. striatus* following standard protocol using the TRIzol reagent (Invitrogen, Scotland, UK) as described elsewhere (Mohapatra & Barman, 2014; Chakrapani *et al*., 2016). RNA extracts were treated with DNase I so as to ensure it is free from DNA contamination. The precipitated RNA extracts were suspended into diethyl pyrocarbonate-treated (DEPC) water. The mRNA was isolated by using mRNA purification kit (SigmaAldrich, St. Louis, MO, USA) following manufacturer's instructions. The quantity estimation and quality assessment were carried out by spectrophotometric readings and agarose gel electrophoresis containing formamide. PCR-Select Subtraction Kit (Clontech, Mountain View, CA, USA) was utilized to construct an SSH cDNA library as described (Barman, Panda, Mohapatra, Swain, & Eknath, 2011; Barman *et al*., 2012) with minor modifications. Briefly, cDNA templates were prepared by reverse transcription from 1.5 µg of the pooled mRNA (2 individuals from each triplicate of 39, 45, 47 and 61 days treatments of independent hypoxic and normoxia group) using Mint cDNA synthesis Kit (Evrogen, Moscow, Russia). SSH libraries were constructed using normoxic cDNAs as driver, while hypoxic counterparts as tester. Both tester and driver cDNAs were independently digested with *Rsa*I. Tester cDNA was ligated with adapter molecules, which are supplied with the above kit. Normalization and enrichment of the differentially expressed cDNAs were performed by hybridization followed by desired PCR amplifications. Subtractive efficiency was validated by PCR amplifications of subtracted and unsubtracted cDNAs for the housekeeping β-actin gene and other stress-inducible

genes such as NADH dehydrogenase, fatty acid binding protein, heat shock protein 90β (HSP90β) and prefoldin subunit 6. The primers used along with respective annealing temperatures are listed in supplementary Table 1. The cycle numbers are mentioned in Figure 1A. The purified secondary PCR products generated from the forward-subtracted cDNAs were ligated into pGEM®-T Easy Vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* DH5α competent cells. Transformed cells were plated onto agar plates for the purpose of generating subtracted/enriched cDNA library.

DNA Sequencing and Computational Tools

DNA sequencing was performed with the help of an automated ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The sequences were blasted in the BLASTN program (Altschul, Gish, Miller, Myers, & Lipman, 1990). The amino acid sequence was deduced by Expasy translate tool (Gasteiger *et al*., 2003). BLASTP was utilized for the verification of amino acid sequences. The gene ontology (GO) analysis was performed with the help of UniPort database (UniProt, 2011).

Quantification of mRNA Level by Quantitative Real Time PCR (qPCR)

The modulated gene expressions (mRNA levels) induced by hypoxic stress exposure were quantified by qPCR analysis using Light Cycler-480 SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) in a Light Cycler 480 q-PCR instrument (Roche Diagnostics, Mannheim, Germany), as described elsewhere (Mohapatra *et al*., 2010; Panda,

Table 1. List of primers designed for q-PCR and SSH efficiency test

Clone name	Primers	Sequence (5'-3')	Primer length (bp)	Anneling Tempareture (°C)	Amplicon size (bp)
β–actin	Forward	GTATGTGGCCATCCAGGCT	19	58	199
	Reverse	TAGCCACGCTCGGTCAGGAT	20		
NADH dehydrogenase	Forward	CCAATTCGCGCACAGTGGA	19	58	200
(SSHCSHL-20)	Reverse	CCTGGTCTGACGTATGCAGC	20		
Fatty acid binding protein	Forward	AGGAGTTCCTCAAGGCCAT	19	58	209
(SSHCSHL-502)	Reverse	CAGGTTGACTGTGCACTTGA	20		
Prefoldin subunit 6	Forward	TTTCAGCCAGAGTGAAGGCT	20	58	199
(SSHCSHL-436)	Reverse	TCGAACTTGCAGCTACGACA	20		
HSP90β (SSHCSHL-8)	Forward	CTGGAGATCAACCCTGACCA	20	58	199
	Reverse	CGTCGATACCCAGTCCGAGT	20		
3-oxo-5-beta-steroid 4-	Forward	ATGGTTCGCCCAGCTTTGG	19	58	207
dehydrogenase (SSHCSHL-11)	Reverse	CCGGCATCTTTGCAAGCCTC	20		
Apolipoprotein C-I (SSHCSHL-	Forward	TCGTTGCATACACAGAGGCT	20	58	198
240)	Reverse	ACTGGCCGATCTCACCAACC	20		
Apolipoprotein A-I (SSHCSHL-	Forward	GCTGCTACTGTCACCAAGAGC	21	58	294
119)	Reverse	ATGTGTGCACCAAGCATGTCTG	22		
Calmodulin	Forward	CCAGAAAATGACTAATCTTACCATGCT	27	58	234
(SSHCSHL-96)	Reverse	AAATGGAGCATCTTGTCCTCAA	22		
Apolipoprotein14KDa	Forward	AATAATCCACGGGCCTTGTCCA	22	58	268
SSHCSHL-44	Reverse	ATTGGCACTGATCCTCACTCTG	22		
Flavin monooxygenase	Forward	AGAGCACTTCAAACTGCTGC	20	58	207
(SSHCSHL-225)	Reverse	ATTCCTGGGAAGTCTTTGAGCG	22		
C1q-like protein	Forward	TGGCCTGGATAATGCCACAC	20	58	203
(SSHCSHL-24)	Reverse	AAGGGTCCAATGGTTCCACT	20		
Gluthathione S-transferase	Forward	CTCTGCAGAATTTGCACGTGTT	22	58	214
(SSHCSHL-101)	Reverse	GGATACTGGCTGACGTCCAC	20		
Retinol-binding protein	Forward	TACCTGCAGTCTGGAAACGA	20	58	212
(SSHCSHL-281)	Reverse	CTGTATTTGCCGAGCAGACA	20		
Serum amyloid A (SSHCSHL-	Forward	GCGGGTGATATGTGGCAAGC	20	58	143
496)	Reverse	GCATCCCTGAAAACTTCCGCTG	22		
Cytochrome P450	Forward	TGAACATCGCAAACTGGCCTGC	22	58	213
(SSHCSHL-213)	Reverse	GTCATCGTAGGTAAAACGCTGTCC	24		
Uncharacterized	Forward	ACTCGAACGGAGCGATGCAGT	21	58	104
(SSHCSHL-241)	Reverse	TGAGCAGGGAAATCTGTTGGCG	22		
Uncharacterized	Forward	ACTTTGGAGGCCAGTGTGAA	20	58	237
(SSHCSHL-275)	Reverse	TGAAACCACCACTGGAAGACCT	22		
Uncharacterized	Forward	TTTCAGCCAGAGTGAAGGCT	20	58	213
(SSHCSHL-338)	Reverse	TCGAACTTGCAGCTACGACA	20		
Uncharacterized	Forward	AAACACCGGCCTCCCAGCTA	20	58	202
(SSHCSHL-387)	Reverse	TCCAAGGCAAAGTTCAACACCG	22		
Uncharacterized	Forward	TCCAGGTGTCTTAGCCCA	18	58	253
(SSHCSHL-120)	Reverse	AGTCCGTTATCTGAAGCCAGA	21		
Unknown	Forward	ATACAGACCCACCGCAGCAT	20	58	191
(SSHCSHL-529)	Reverse	TGTGAGTGTCCTGTTCAAACGG	22		

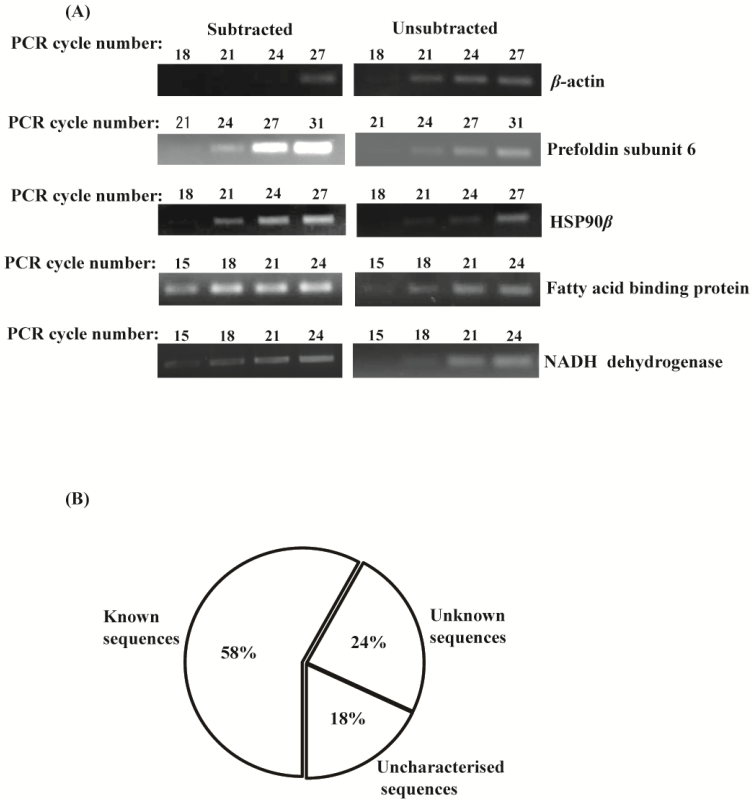

Figure 1. Subtraction efficiency verification and characterization of ESTs.
(A) Subtraction efficiency was estimated by polymerase chain reaction (PCR) amplification of *β*-actin, Prefoldin subunit 6, HSP90 *β*, Fatty acid binding protein and NADH dehydrogenase from subtracted and unsubtracted cDNA libraries. The number of PCR cycles is indicated above each lane. (B) EST classification represented in subtracted library based on sequence analysis of 130 non-redundant inserts. Known sequences exhibit significant homology with known genes. Uncharacterized sequences were homologous to unannotated EST sequences. Sequences with no significant match were called unknown sequences.

Barman, & Mohapatra, 2011; Barman *et al*., 2012; Barman *et al*., 2015; Patra *et al*., 2015). The mRNAs from the livers of ≥5 individuals (from triplicate) were extracted independently from each day (39, 45, 47 and 61 days) of hypoxic and normoxic treatments. After verifications of RNA quality and integrity, mRNA (equal quantity each) was pooled for cDNA preparation. The sequence information of PCR amplified bands was confirmed by sequencing. The most stable house-keeping gene among *β*-actin, glyceraldehyde 3-phosphate dehydrogenase (*G3PDH)*, cytochrome c oxidase subunit I (*CoI*) and cytochrome c oxidase subunit II (*CoII)* was identified using geNorm rankings (Mestdagh *et al*., 2009; Mohanta, Jayasankar, Das Mahapatra, Saha, & Barman, 2014; Mohapatra *et al*., 2014). Briefly, relative expression level of candidate house-keeping genes were calculated in four different tissues such as brain, heart, liver and muscle (from normoxic and hypoxic fishes). The expression stability values (M) for each gene were estimated with the help of geNorm software, where a lower 'M' value corresponding to more stable gene expression. The stability patterns were similar for normoxic and hypoxic treatments. Hence, data from both the treatments were combined as reported earlier (McCurley & Callard, 2008). *β*-actin was ranked as the most stable gene (Figure 2) and hence used as the internal control for the purpose of normalization to estimate relative transcript levels of target genes. Primer annealing temperature for target genes as well as *β*-actin was 58°C. Primers are enlisted in Table 1. A simultaneous PCR reaction using RNA as a template with *β*-actin primer set (as a negative control) was carried out to rule out the possibility of DNA contamination. The expression data obtained were subjected to one-way analysis of variance (one-way ANOVA) followed by an unpaired two-tailed T-test (Panda *et al*., 2014). The $P<0.05$ was considered statistically significant. All data were represented as mean ±SE.

Rapid Amplification of cDNA ends (RACE)

RACE-PCR was performed to obtain the 5′- and 3′-ends of the SSH generated ESTs (SSHCSHL-8, SSHCSHL-20, SSHCSHL-338) using Smarter RACE cDNA amplification Kit (Clontech, USA) following protocol as described (Barman *et al*., 2012). Gene-specific primers (GSP1 and GSP2) designed from the generated sequence data are enlisted in Table 2. GSP1 and Universal Primer A mix (UPM, provided with the kit) were used for conducting the first PCR (touch-

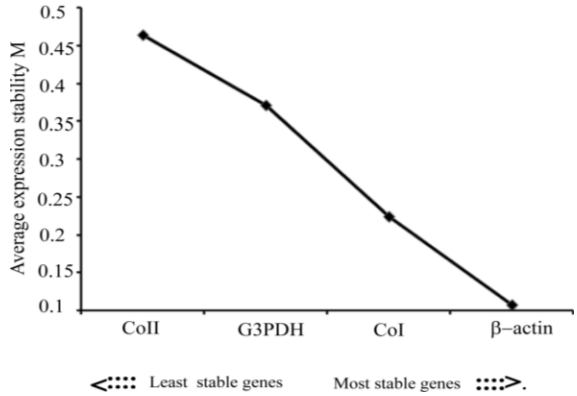

Figure 2. geNorm expression stability plot. Avearge expression stability value of control genes by geNorm rankings indicating the degree of variability between the least and most stable genes in a different tissue panel. β-actin, Glyceraldehyde 3-phosphate dehydrogenase (G3PDH), Cytochrome c oxidase subunit I (CoI) and Cytochrome c oxidase subunit II (CoII). β-actin was identified as the most stable gene.

Table 2. List of gene specific primers (GSP) designed for RACE-PCR

Clone name	RACE primer	Sequence (5′- 3′)	Size (base)	Tm (°C)
HSP90β (SSHCSHL-8)	GSP1 (5′-RACE)	ACAGCCTTGTCGTTCTTGTCAGCTTCAG	28	62.2
	GSP2 (5′-RACE)	TGGTCAGGGTTGATCTCCAGATGC	24	60.4
	GSP1 (3′-RACE)	TCATGAAGGCCCAGGCACT	19	58.6
	GSP2 (3′-RACE)	GAGGCCACCTCTACAGCTGTCCCAG	25	64.4
NADH dehydrogenase	GSP1 (5′-RACE)	GTGGTTCAGGATGCGAGTCAGC	22	60.2
(SSHCSHL-20)	GSP2 (5′-RACE)	ACTGAGCAACTTCTCCACAGCCA	23	60.1
	GSP1 (3′-RACE)	ACGCTGCATACGTCAGACCAGGTGG	25	64.2
	GSP2 (3′-RACE)	GATGGATGACATCTACGAGTGGTGCA	26	59.9
Uncharacterised (SSHCSHL-338)	GSP1 (5′-RACE)	ATGCCTGGCTGATTCAAACGTGAGCA	26	62.6
	GSP2 (5′-RACE)	AGTCCCATGATGACATGCAACCCTTGGA	28	63.1

down) with amplification parameters: initial 5 cycles of 30 sec denaturation at 94°C, 3 min annealing at 64° C for 3′-end but 70°C for 5′-end extensions; subsequent 5 cycles of 30 sec at 94°C, 30 sec at 62°C for 3′-end but 68°C for 5′-end, 3 min at 72°C; and final 25 cycles of 30 sec at 94°C, 30 sec at 60°C for 3′-end but 66°C for 5′-end, and 3 min extension at 72°C. Fifty times diluted first PCR products were subjected to the second round of PCR using GSP2 and Nested Universal Primer A (NUP, provided with the kit) with cycling parameters of 25 cycles of 30 sec at 94°C, 30 sec at 60°C for 3′-end but 66°C for 5′-end, and extended for 3 min at 72°C. The desired bands from second PCR products were gel-extracted (1.5% agarose) using gel extraction kit (USB, Fountain Valley, CA USA), cloned into pGEM®-T easy vector (Promega, Madison, WI, USA), transformed into chemically competent DH5α cells, and bi-directionally sequenced as stated above.

Results

Enrichment of cDNA by SSH Library Construction from the Liver of Hypoxic *C. striatus* and Assembly of ESTs

C. striatus was imposed with hypoxia stress inside the laboratory, for a period of 61 days, based on the protocol described earlier (Mohapatra *et al*., 2010). To identify the genes associated with adaptability to hypoxic condition, a forward-subtracted cDNA library was constructed from the pooled mRNA extracted from the liver of *C. striatus* under hypoxic stress, representing tester cDNA and normoxic exposure as a driver. The SSH generated enriched transcripts, which are likely to be the representative hypoxia-induced.

Subtraction efficiency was examined by comparing the removal of housekeeping β-actin gene, while enriching known stress-inducible genes between the templates of subtractive and unsubtractive PCR products of the second round (Figure 1A). β-actin transcript could be detected with only 21 cycles of PCR amplification in the un-subtracted library as compared to 27 cycles for subtracted library. Contrary to this, known stress-inducible (including hypoxia stress) genes such as those encoding prefoldin, HSP90β, fatty acid binding protein and NADH dehydrogenase (Heads, Yellon, & Latchman, 1995; Almgren & Olson, 1999; Davidson & Schiestl, 2001; David *et al*., 2005; Wang *et al*., 2005; Rajaraman *et al*., 2007; Rodriguez-Milla & Salinas, 2009; Woo, Jeon, Kim, & Yum, 2011) were amplified in early PCR cycles in subtracted library than unsubtracted one (Figure 1A). Evidences of up-regulation (mRNA) of these known genes, from our experimental (hypoxic) fishes, by quantitative real time PCR (qPCR) analysis are also provided in a later

section (Figure 3). Simultaneous reduction in *β*-actin gene cDNA and increase in stress-inducible counterparts to subtracted liver cDNA indicated that a large number of constitutive transcripts were removed effectively, while hypoxia-induced ESTs were enriched efficiently.

The PCR products of SSH were cloned into TA-cloning vector. In total, 204 randomly picked clones contained inserts. All the clones bearing inserts were bi-directionally sequenced. Insert-size ranged from about 60 bp to 1230 bp. Of the 204 clones sequenced, 146 good quality sequences were aligned with those in the GenBank databases and submitted to GenBank (GenBank Accession Library Name: LIBEST_027526, *Channa striatus* liver library). Among them, ten clones belonged to the ribosomal proteins; while six clones were redundant types. The redundant clone CSHLSSH44, encoding apolipoprotein, repeated four times. Transcripts of CSHLSSH5 (encoding complement component) and CSHLSSH99 (chymotrypsin) each repeated three times, while CSHLSSH40 (for cadherin), CSHLSSH250 (translable to aldolase) CSHLSSH473 (encoding succinate dehydrogenase iron-sulfur subunit) were redundant twice. Enrichment of certain transcripts encoding ribosomal proteins in SSH enriched cDNA library was previously evidenced (Barman *et al*., 2011). Because SSH cDNA library is PCR based method, the possibility of generating redundant clones are expected.

The BLAST results for rest 130 transcripts are shown in Figure 1B. Of these non-redundant sequences, 32 clones (24%) exhibited no significant homology to any previously identified genes (termed unknown). About 58% (75 clones) of putative transcripts showed significant (>70%) sequence homology with other vertebrate genes (termed known) as shown in Table 3, while 23 EST fragments (18%) were homologous either to genes with unknown function or to unannotated ESTs (termed uncharacterized) as summarized in Table 4. However, many uncharacterized transcripts matched with fish ESTs available in the public domain (Table 4). Many clones of known category (Table 3) matched with stress-induced ESTs particularly linked to hypoxia tolerance for fish species. As shown in Figure 4, known transcripts represented broad spectrum of biological pathways (http://www.uniprot.org/help/gene_ontology by EMBL-EBI) such as transcription/translation factors, signal transductions, energy metabolisms, electron transports, immune responses, proteolytic processes, reproductive cycles, transport-facilitators, etc. Together, these results validated that enriched transcripts participating complex biological processes were enriched and those are likely to be linked with hypoxia tolerance.

Relative expression Patterns of ESTs in the Liver of *C. striatus* Imposed with Hypoxia Stress

To confirm the outcomes of SSH-mediated enrichment of hypoxia-induced transcripts, 21 selected genes were analyzed by qPCR to quantify their mRNA abundances in the liver. The criteria of selecting known EST clones for qPCR analysis were based on earlier evidences associated with stress tolerances (representing a variety of functional

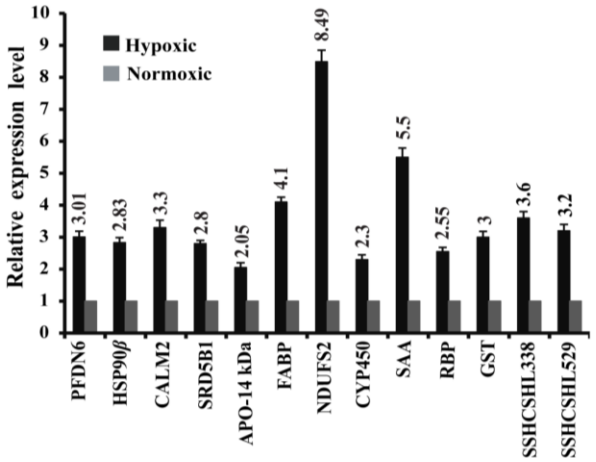

Figure 3. The expression profiles of selected ESTs enriched from SSH cDNA library by quantitative real time polymerase chain reaction (qPCR). The qPCR data for all ESTs was normalized with *β*-actin as reference gene. The qPCR data shows the relative gene expression levels in the hypoxia-stressed *C. striatus* liver tissue over normoxic counterparts. An elevated mRNA levels for NADH dehydrogenase iron-sulfur protein 2 (SSHCSHL-20), Serum amyloid A (SSHCSHL-496), Fatty acid binding protein (SSHCSHL-502), respectively, by 8.5-, 5.5- and 4.1-fold, were documented in hypoxic liver. The rest of the clones were up-regulated in the tune of ≥2 folds. The numbers on top of each bar represent fold-changes in expressions in hypoxia relative to the expressions in normoxic fish. The data represent the average of three independent qPCR experiments (each in triplicate) ($P<0.05$). PFDN6, Prefoldin subunit 6; HSP90*β*, Heat shock protein 90*β*; CALM2, Calmodulin 2; SRD5B1, 3-oxo-5-beta-steroid 4 dehydrogenase; APO-14 kDa, Apolipoprotein14 kDa; FABP, Fatty acid binding protein; NDUFS2, NADH dehydrogenase iron-sulfur protein 2; ; CYP450, Cytochrome P450; FMO2, SAA, Serum amyloid A protein; RBP, Retinol-binding protein; GST, Gluthathione S-transferase; SSHCSHL338, Uncharacterized; SSHCSHL529, Unknown.

Table 3. The SSH-generated cDNA clones showing significant similarity to known sequences in the public databases

Clone name	AccessionNo	Gene name	Species	E-value	Percentage of Homology
Regulation of transcription and translation					
SSHCSHL-106	JK546335.1	Eukaryotic translation initiation factor 3	*Anoplopoma fimbria*	1e-13	90%
SSHCSHL-318	JK546405.1	Eukaryotic translation elongation factor 2b (eef2b)	*Danio rerio*	2e-31	86%
SSHCSHL-390	JK546418.1	C-myc binding protein	*Scophthalmus maximus*	4e-55	87%
SSHCSHL-436	JK546427.1	Prefoldin subunit 6	*Salmo salar*	7e-102	80%
SSHCSHL-466	JK546435.1	Eukaryotic translation initiation factor 4A, isoform 2 (eif4a2)	*Danio rerio*	6e-44	85%
SSHCSHL-505	JK546445.1	eukaryotic translation elongation factor 2	*Danio rerio*	1e-102	85%
SSHCSHL-509	JK546448.1	Cysteinyl-tRNA synthetase	*Platichthys flesus*	2e-22	74%
Cellular signaling					
SSHCSHL-8	JK546311.1	Heat shock protein 90β	*Pagrus major*	0.0	84%
SSHCSHL-17	JK546316.1	Complement regulatory plasma protein	*Paralabrax nebulifer*	2e-15	74%
SSHCSHL-40	JK546326.1	Cadherin 2	*Rattus norvegicus*	6e-07	68%
SSHCSHL-96	JK546330.1	Calmodulin 2	*Salmo salar*	1e-59	82%
SSHCSHL-118	JK546343.1	Peptidyl-prolyl cis-trans isomerase B	*Salmo salar*	2e-43	75%
SSHCSHL-121	JK546346.1	Transcript variant 1	*Danio rerio*	6e-57	75%
SSHCSHL-220	JK546357.1	Insulin-like growth factor I	*Paralichthys olivaceus*	2e-54	77%
SSHCSHL-224	JK546361.1	Casein kinase 2	*Salmo salar*	4e-95	91%
SSHCSHL-286	JK546390.1	Reticulon-1-A	*Anoplopoma fimbria*	3e-43	91%
SSHCSHL-306	JK546400.1	Heat shock protein 90	*Epinephelus coioides*	2e-72	87%
SSHCSHL-341	JK546411.1	Heat shock protein 90β (grp94), member 1 (hsp90b1)	*Danio rerio*	8e-64	93%
SSHCSHL-492	JK546440.1	C1R/C1S subunit of Ca2+-dependent complex	*Oncorhynchus mykiss*	2e-19	81%
Metabolic pathway					
SSHCSHL-11	JK546313.1	3-oxo-5-beta-steroid 4 dehydrogenase	*Anoplopoma fimbria*	0.0	84%
SSHCSHL-13	JK546453.1	F-acid glycoprotein	*Neoditrema ransonnetii*	1e-29	68%
SSHCSHL-26	JK546319.1	Apolipoprotein A-I precursor	*Anoplopoma fimbria*	4e-13	78%
SSHCSHL-273	JK546381.1	Apolipoprotein A-I precursor	*Epinephelus coioides*	6e-68	79%
SSHCSHL-44	JK546329.1	Apolipoprotein 14 kDa	*Oplegnathus fasciatus*	2e-70	77%
SSHCSHL-119	JK546344.1	Apolipoprotein A-I	*Oplegnathus fasciatus*	3e-163	83%
SSHCSHL-209	JK546349.1	Phosphoenolpyruvate carboxykinase	*Lateolabrax japonicus*	1e-30	74%
SSHCSHL--240	JK546370.1	Apolipoprotein C-I	*Solea senegalensis*	3e-23	87%
SSHCSHL-250	JK546373.1	Aldolase B	*Perca flavescens*	2e-19	74%
SSHCSHL-293	JK546395.1	Carboxyl ester lipase	*Danio rerio*	5e-34	76%
SSHCSHL-374	JK546415.1	Apolipoprotein 14 kDa	*Perca flavescens*	7e-38	73%
SSHCSHL-400	JK546420.1	Lecithin-cholesterol acyltransferase (LCAT)	*Bos taurus*	1e-16	75%
SSHCSHL-440	JK546429.1	Uridine phosphorylase 2 (Upp2)	*Mus musculus*	7e-26	76%
SSHCSHL-501	JK546443.1	Carboxypeptidase	*Oreochromis niloticus*	1e-83	78%
SSHCSHL-502	JK546444.1	Fatty acid binding protein	*Sparus aurata*	9e-151	84%
Electron transport chain					
SSHCSHL-20	JK546317.1	NADH dehydrogenase iron-sulfur protein 2	*Anoplopoma fimbria c*	8e-176	91%
SSHCSHL-33	JK546323.1	Uncoupling protein 1 ((UCP1)	*Siniperca chuatsi*	5e-166	86%
SSHCSHL-213	JK546353.1	Cytochrome P450 2R1	*Danio rerio*	1e-92	71%
SSHCSHL-225	JK546362.1	Flavin-containing monooxygenase L2	*Oncorhynchus mykiss*	6e-28	79%
SSHCSHL-309	JK546401.1	Cytochrome P450, family 8, subfamily B, CYP8B1	*Platichthys flesus cDNA*	4e-90	71%
SSHCSHL-331	JK546408.1	Cytochrome oxidase subunit I (COI)	*Channa striata*	6e-38	91%
SSHCSHL-473	JK546447.1	Succinate dehydrogenase iron-sulfur subunit	*Anoplopoma fimbria*	3e-38	78%
SSHCSHL-532	JK545451.1	Cytochrome c oxidase subunit I (COXI)	*Danio rerio*	3e-113	77%
Immune response					
SSHCSHL-5	JK546308.1	Complement component C3	*Paralichthys olivaceus*	1e-41	80%
SSHCSHL-15	JK546314.1	Rhamnose-binding lectin (RBL)	*Channa argus*	6e-101	84%
SSHCSHL-24	JK546318.1	C1q-like 23kDa protein	*Neoditrema ransonnetii*	2e-40	70%
SSHCSHL-97	JK546331.1	Serum amyloid P	*Anoplopoma fimbria*	6e-60	80%
SSHCSHL-109	JK546338.1	MHC class I antigen (Onmy-UBA)	*Oncorhynchus mykiss*	6e-50	76%
SSHCSHL-221	JK546358.1	Complement component C3	*Paralichthys olivaceus*	3e-118	77%
SSHCSHL-235	JK546368.1	Endonuclease, polyU-specific (ENDOU)	*Bos taurus*	5e-28	74%
SSHCSHL-300	JK546396.1	MHC class I alpha antigen	*Epinephelus akaara*	7e-60	88%
SSHCSHL-432	JK546426.1	Immunoglobulin M heavy chain	*Channa argus*	4e-108	86%
SSHCSHL-496	JK546442.1	Serum amyloid A protein	*Fundulus heteroclitus*	2e-37	72%
Proteolytic Processes					
SSHCSHL-9	JK546312.1	Trypsinogen 3	*Paralichthys olivaceus*	1e-115	85%
SSHCSHL-37	JK546324.1	Chymotrypsinogen II precursor	*Sparus aurata*	8e-16	76%
SSHCSHL-39	JK546325.1	Choriolytic enzyme 1	*Anoplopoma fimbria*	2e-59	89%
SSHCSHL-99	JK546332.1	Chymotrypsinogen B1	*Danio rerio*	1e-124	79%
SSHCSHL-114	JK546340.1	Chymotrypsin-like protease CTRL-1	*Anoplopoma fimbria*	2e-69	85%
SSHCSHL-216	JK546354.1	Serpin	*Oreochromis niloticus*	0.0	78%
SSHCSHL-230	JK546364.1	Alpha-1-antitrypsin	*Epinephelus coioides*	9e-168	77%
SSHCSHL-251	JK546374.1	Trypsinogen 3	*Solea senegalensis*	5e-109	86%
SSHCSHL-269	JK546379.1	Elastase 1	*Paralichthys olivaceus*	1e-37	79%
SSHCSHL-375	JK546416.1	Trypsin	*Siniperca chuatsi*	1e-35	91%
SSHCSHL-391	JK546419.1	Trypsinogen Y	*Solea senegalensis*	8e-51	87%
SSHCSHL-409	JK546423.1	Serine/Cysteine proteinase inhibitor	*Epinephelus coioides*	3e-48	71%
Reproductive pathway					
SSHCSHL-30	JK546322.1	Vitellogenin B	*Morone americana*	7e-160	77%
SSHCSHL-212	JK546352.1	Vitelline membrane outer layer protein 1 (vmo1)	*Salmo salar*	2e-06	79%
SSHCSHL-281	JK546389.1	Retinol-binding protein	*Epinephelus coioides*	6e-81	87%
SSHCSHL-312	JK546402.1	Vitellogenin B	*Morone americana*	0.0	83%
SSHCSHL-444	JK546430.1	Choriogenin H	*Fundulus heteroclitus*	7e-70	76%
Cellular transportation					
SSHCSHL-101	JK546333.1	Gluthathione S-transferase	*Takifugu obscurus*	5e-102	80%
SSHCSHL-107	JK546336.1	Transferrin	*Epinephelus coioides*	1e-27	80%
SSHCSHL-214	JK546309.1	Transferrin	*Pagrus major*	2e-62	74%

Table 3. Continued

Clone name	AccessionNo	Gene name	Species	E-value	Percentage of Homology
Regenerative pathway					
SSHCSHL-228	JK546363.1	Myelin-associated glycoprotein, Precursor	*Salmo salar*	5e-10	77%
SSHCSHL-285	JK546389.1	Chemotaxin (lect2 gene)	*Pseudosciaena crocea*	9e-106	82%
Angiogenesis					
SSHCSHL-401	JK546421.1	Angiopoietin-like 3 (angptl3)	*Danio rerio*	4e-15	69%

Table 4. Identified transcripts showing significant homology with unannotated ESTs

Clone number (length)	% identity (length)	e-value	Species	GenBank acc. no.	Organ
SSHCSHL-29(545bp)	142/181 (78%)	6e-33	*Thunnus thynnus*	EC918383.1	Adult testis
SSHCSHL-120(772bp)	438/600 (73%)	3e-88	*Paralichthys olivaceus*	CX286648.1	Liver
SSHCSHL-113(399bp)	352/424 (83%)	4e-115	*Dissostichus mawsoni*	FE198756.1	Adult brain
SSHCSHL-115(537bp)	411/547 (75%)	3e-100	*Siniperca chuatsi*	GR478862.1	Muscle
SSHCSHL-122(258bp)	209/260 (80%)	3e-57	*Platichthys flesus*	DV569512.1	Liver
SSHCSHL-223(266bp)	152/191 (80%)	1e-31	*Anoplopoma fimbria*	GO640640.1	Mixed tissue
SSHCSHL-233(788bp)	542/718 (75%)	4e-126	*Dissostichus mawsoni*	FE197097.1	Adult brain
SSHCSHL-237(453bp)	221/330 (67%)	9e-17	*Dicentrarchus labrax*	GD180840.1	Liver
SSHCSHL-241(502bp)	145/186 (78%)	5e-33	*Dissostichus mawsoni*	FE217739.1	Liver
SSHCSHL-271(247bp)	135/162 (83%)	2e-35	*Platichthys flesus*	DV568733.1	Liver
SSHCSHL-274(219bp)	138/181 (76%)	2e-27	*Perca fluviatilis*	DY615306.1	Liver
SSHCSHL-275(330bp)	167/196 (85%)	2e-55	*Dicentrarchus labrax*	FK941627.1	Liver
SSHCSHL-301(344bp)	311/352 (88%)	5e-120	*Anoplopoma fimbria*	GO638666.1	Mixed tissue
SSHCSHL-338(400bp)	206/277 (74%)	1e-39	*Dicentrarchus labrax*	FL487096.1	Liver
SSHCSHL-347(289bp)	189/242 (78%)	3e-46	*Perca flavescens*	FM026982.1	Brain
SSHCSHL-387(429bp)	275/418 (66%)	3e-22	*Paralichthys olivaceus*	AU260699.1	kidney
SSHCSHL-402(281bp)	172/241 (71%)	2e-23	*Sebastes caurinus*	GE818215.1	Mixed tissue
SSHCSHL-429(359bp)	180/239 (75%)	1e-33	*Oreochromis niloticus*	GR643982.1	Gill
SSHCSHL-464(330bp)	300/337 (89%)	2e-117	*Anoplopoma fimbria*	GO631191.1	Mixed tissue
SSHCSHL-494(218bp)	101/113 (89%)	2e-33	*Anoplopoma fimbria*	GO622853.1	Mixed tissue
SSHCSHL-507(348bp)	279/347 (80%)	3e-84	*Oreochromis niloticus*	GR642539.1	Gill
SSHCSHL-508(269bp)	211/312 (68%)	2e-29	*Oreochromis niloticus*	GR610512.1	brain
SSHCSHL-519(428bp)	124/147 (84%)	2e-36	*Perca flavescens*	GO654013.1	Ovary

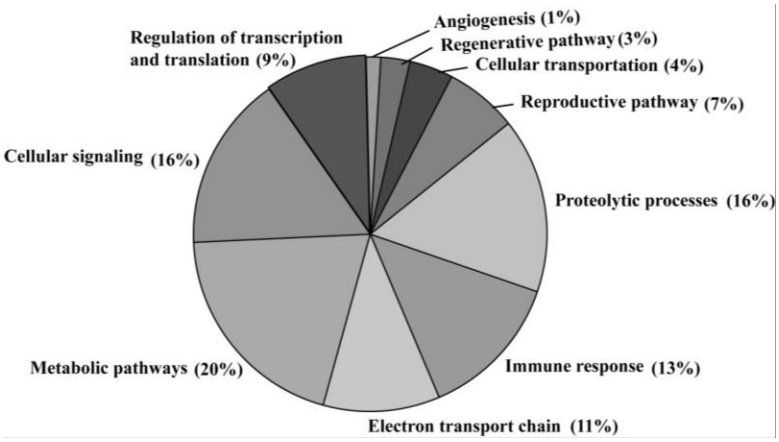

Figure 4. Classification of the known ESTs according to their predicted functions in response to hypoxia. Gene ontology annotation (GO) is used to analyze the predicted biological function of these known gene.

groups) in other species, including fish/crustacean, and quality of the sequence generated by SSH.

Figure 3 shows the fold-change in liver obtained for hypoxia imposition over control (normoxic). Genes exhibiting ≥2-fold change is commonly being considered as the limit of significant differential expressions using qPCR analyses (Morey, Ryan, & Van Dolah, 2006; Sussarellu, Fabioux, Le Moullac, Fleury, & Moraga, 2010). The statistically significant p values ($P<0.05$) of each are shown in supplementary Table 5. The significantly elevated levels of mRNA expressions (≥2-fold increase) were detected in hypoxia-exposed liver than control with NADH dehydrogenase iron-sulfur protein 2 (SSHCSHL-20) and serum amyloid A (SSHCSHL-496) genes by 8.5- and 5.5-fold, respectively. Similar trends of mRNA overexpression were detected for the rest of the clones belonging to known genes in the tune of ≥2-folds. The increased levels of mRNA expressions were documented for unknown and uncharacterized transcripts (Figure 3). Thus, these clones, being up-regulated in the liver of *C. striatus* exposed to hypoxic condition, could be considered as novel ESTs that are most likely to be linked with hypoxia-stress

tolerance. HSP90 was known to be up-regulated during hypoxia stress in rat and human (Almgren & Olson, 1999; Trisciuoglio *et al*., 2010). As expected, HSP90*β* expression was up-regulated in the liver of hypoxic fishes (Figure 3). These findings suggested their possible physiological significance with regard to hypoxia linked adaptive mechanistic functions in *C. striatus*.

Generation and Analysis of Full-Length cDNA Sequences Linked with Hypoxia Stress Tolerance

Attempts were made to generate full length cDNA sequence information of upregulated transcripts during hypoxia exposure. The full-length sequences of prefoldin and fatty acid binding protein (GenBank Accession No. KJ867524 and KJ867523) was obtained from the respective single clone of SSH generated cDNA library. The full-length cDNA sequence of HSP90*β* (KJ867519) was successfully derived by 5′- and 3′-RACE-PCR. Similarly, full-length cDNA for CSHL-338 clone (uncharacterized EST, KJ867525), being up-regulated during hypoxia stress, was also generated.

The full-length sequences are shown in Figure 5. Every known EST contained an open reading frame (ORF) of different lengths with an ATG (M) as start codon and either TGA or TAA as a stop codon. The start codon for cDNAs of fatty acid binding protein and prefoldin were within the consensus sequence based on the Kozak criteria (A/GNNATGG) (Kozak, 1991), while rests showed modified sequences. All the sequences consisted of 5′-flanking region, relative to start codon; and 3′-UTR of variable sizes (Figure 5). The poly-A tail was also identified within 3′-untranslated tail. The consensus polyadenylation signal sequence (Tian, Hu, Zhang, & Lutz, 2005) was identified in cDNAs of HSP90*β* and fatty acid binding protein.

The deduced amino acid profile for each of cDNAs are also depicted in Figure 5. The conserved domains of each of these proteins were predicted by CD-Search (Marchler-Bauer & Bryant, 2004). The prefoldin contained beta catalytic motifs. As expected, histidine kinase-like ATPase (Glu-36 to Leu-184) and subunit-90 (Leu-298 to Asp-708) domains were detected from HSP90*β*. The important domain, such as lipocalin domain (Asn-4 to Thr-114) was identified in fatty acids binding protein. Similarly, Ly-6 antigen (uPA receptor -like domain) is present in the uncharacterized transcript (SSHCSHL-338). These motifs are likely to play significant regulatory roles either independently or co-operatively as binding platforms with other molecules so as to mitigate hypoxia stress.

Discussion

Out of 75 enriched ESTs, 93% was known to be involved in hypoxia stress tolerance by regulating different biological processes such that of protein synthesis, signal transduction, metabolism, transport-facilitators, cell defense proteolysis and reproductive cycle, etc. In acute hypoxia, mitochondria have been implicated as an early respondent by releasing reactive oxygen species (ROS), which in turn triggers a cascade of events involving stabilization of hypoxia-inducible factor (HIF-1). Uncoupling protein 1 (UCP1) is important for the protection against ROS in chronic hypoxia (Marques *et al*., 2008**).** Translational transcripts such as initiation factors (translation initiation factor 3, eukaryotic translation initiation factor 4A) and EF2 were enriched in our SSH library. Elevated translational factors were also documented in response to hypoxia in plants, blue crab, pacific oyster and zebrafish (Hochachka & Lutz, 2001; Brown-Peterson *et al*., 2005; David *et al*., 2005; Marques *et al*., 2008). These are likely to be required for restoration of protein synthesis.

Cell signaling governs basic cellular activities for coordinated cell actions. Several transcripts linked to this particular biological function were also enriched in this library (Table 3). Among these, casein kinase 2, an important regulator of HIF-1, is a well-known player for the signaling pathway controlling the hypoxic adaptation (Mottet, Ruys, Demazy, Raes, & Michiels, 2005). Insulin growth factor I (IGFI), being a pleiotropic anabolic growth

Table 5. P values of significantly upregulated genes selected from qRT-PCR

Gene Name	Fold increase	Standard Deviation	P value
PFS6	3.01	0.31	0.000381651
HSP90*β*	2.83	0.27	0.000339389
CALM2	3.3	0.40	0.000628685
SRD5B1	2.8	0.18	7.06179E-05
APO-14 kDa	2.05	0.25	0.002155749
FABP	4.1	0.26	3.48065E-05
NDUFS2	8.49	0.6	3.08134E-05
CYP450	2.3	0.26	0.00104563
SAA	5.5	0.5	0.000123636
RBP	2.55	0.23	0.000321774
GST	3	0.2	0.000394286
SSHCSHL338	3.6	0.37	0.000300894
SSHCSHL529	3.2	0.35	0.000429456

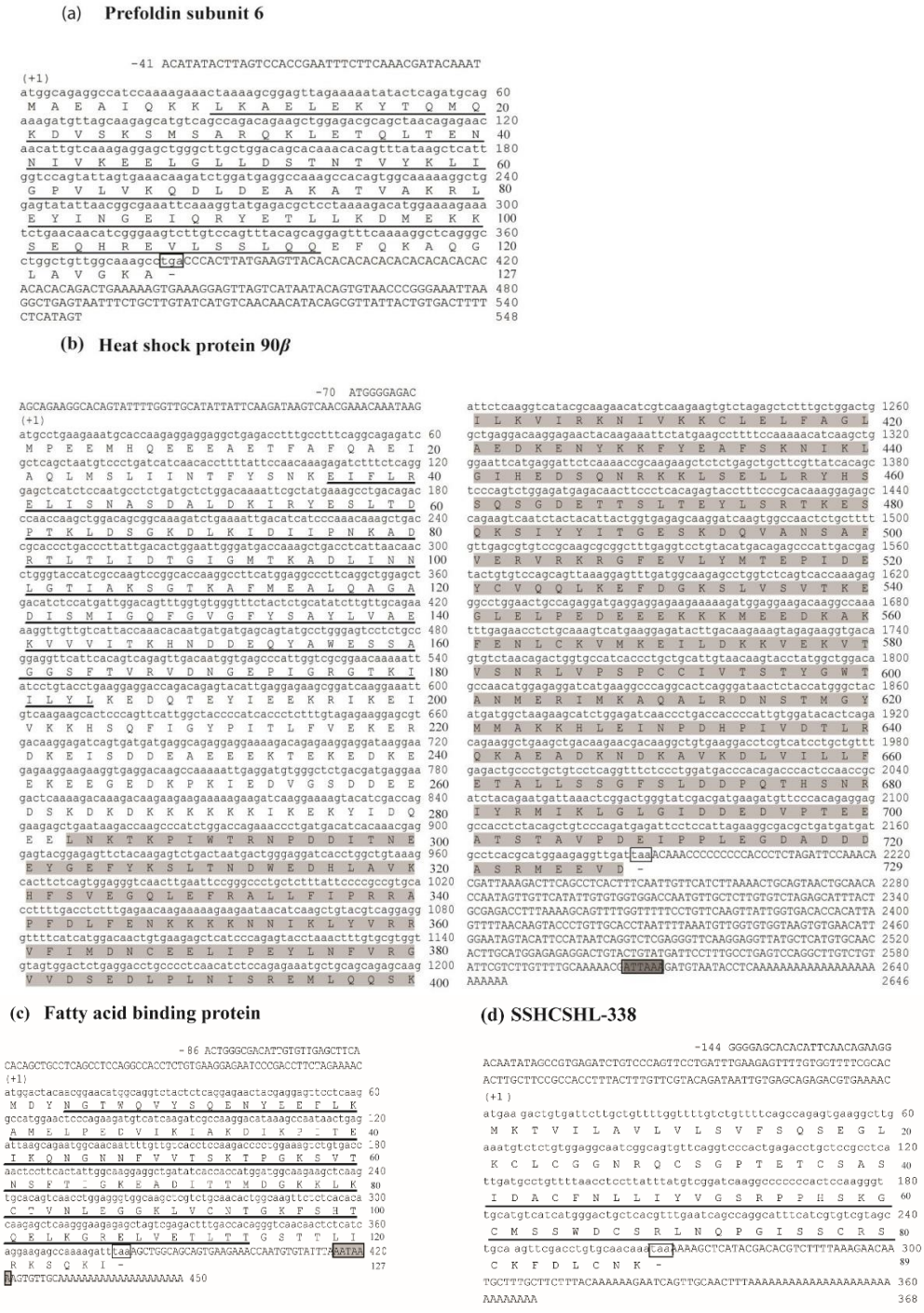

Figure 5. Generation of the full length cDNA sequences and their deduced amino acid sequences. (a**)** Prefoldin subunit 6 (SSHCSHL-436), (b) Heat shock protein 90*β* (SSHCSHL-8), (c) Fatty acid binding protein (SSHCSHL-502), (d) An uncharacterised EST (SSHCSHL-338). The ORF is shown in small letter. The in-frame stop codon and polyadenylation signal is marked within the white and grey-boxes, respectively. The 5′- and 3′-UTRs are shown in capital letters. The identified domains as mentioned in results are underlined and grey-shaded.

factor partially activating HIF-1, promotes neuronal survival as a mode of hypoxia tolerance during hypoxic-ischemic injury (Wang, Deng, Boyle, Zhong, & Lee, 2004b). Calmodulin is known to transduce calcium signals by binding with calcium ions, and subsequently proving the platforms for other interacting molecules of downstream signals. It's increased expression was evidenced in hypoxic rat (Zhao, Pan, Li, & Sun, 2008)**.** Thus, cell signaling pathways play a pivotal role in mitigating hypoxia stress.

ESTs, associated with metabolic pathways, were activated during hypoxic stress. Transcripts of adlolase, 3-oxo-5-beta-steroid 4 dehydrogenase and PEPCK were enriched. Evidences are available with regard to aldolase, containing HIF-1 binding site, mediates glycolytic pathway (Semenza *et al*., 1996; Marques *et al*., 2008). The enzyme 3-oxo-5-beta-

steroid 4 dehydrogenase participates controlling pathways of bile acid biosynthesis and steroid hormone metabolism. A reduction in oxidative ATP formation leads to an increase of non-oxidative energy production mediated by glycolytic pathway in hypoxic tissue. The enrichment of ESTs encoding metabolic enzymes demonstrated a shift from aerobic to anaerobic metabolism induced by hypoxia.

One of the important functions of oxidative stress tolerance has been regulated by the electron transport chain. HIF-1 controls the metabolic adaptations by activating transcription of the genes encoding COX4-2 (cytochrome c oxidase) during hypoxic condition (Semenza, 2007). In anaerobic conditions, cells utilize ethanol produced during the glucose fermentation. Ethanol is preferentially oxidized to acetaldehyde by cytosolic alcohol dehydrogenase, forming NADH in the process. This results in the rise of cytosolic NADH/NAD ratio. The excess NADH is oxidized by the mitochondrial respiratory chain via NADH dehydrogenase, located on the inner mitochondrial membrane (Davidson & Schiestl, 2001) Flavin-containing monooxygenase, Cytochrome P450 and Cytochrome c oxidase subunit I (COXI) were reported as up-regulated in response to hypoxia (David *et al*., 2005; Baze, Schlauch, & Hayes, 2010). The HIF-1 binding motif is present in monooxygenase (Shen, Nettleton, Jiang, Kim, & Powell-Coffman, 2005; Sugimoto *et al*., 2008). Prolonged oxidative stress causes pathogenesis of most chronic diseases. That may be the reason that several defense related genes were expressed and hence those transcripts were enriched in the liver of hypoxic *C. striatus*. Among these, complement component 3, playing a central role in the complement system linked to innate immunity, was up-regulated during hypoxia treatment (Marques *et al*., 2008; Bauer *et al*., 2011). C1q protein, a subcomponent of the complement system, exhibited heightened expression in hypoxia exposed rat PC12 Cells (Tohgi, Utsugisawa, & Nagane, 2000). The enrichment and documented up-regulation of serum amyloid A is in line with previous findings that a rise in SAA protein in response to hypoxic ischemia (Aly *et al*., 2011).

Genes of serpin superfamily are believed to play important roles of inhibiting proteolytic and associated cascading reactions, those could otherwise cause cumulative damages to energy-restricted tissues over a period of time (Storey, 2004). Heightened mRNA expressions for α-1-antitrypsin and serine/cysteine proteinase inhibitors, belonging to serpin family, were also documented in zebrafish during hypoxic exposure (Marques *et al*., 2008).

Transferrin and gluthathione S-transferase are ion transporters. These genes were also highly expressed in hypoxic condition (Rolfs, Kvietikova, Gassmann, & Wenger, 1997; Tacchini, Bianchi, Bernelli-Zazzer, & Cairo, 1999; Marques *et al*., 2008). Thus, hypoxia induces ion-transport mechanism by influencing cellular transportation proteins. Angiopoietin-like 3 (ANGPTL3) plays a role in the regulation of angiogenesis. It is predominantly expressed in the liver. Its heightened mRNA expression due to hypoxia challenge in rat liver was documented (Abdulmalek *et al*., 2001)

Our findings revealed that hypoxia stress adaptive response involved induction of a set of genes. Among these, we have generated full-length cDNA sequence information of selected up-regulated known transcripts (HSP90*β*, fatty acid binding protein and prefoldin subunit 6). Additionally, an uncharacterized EST (SSHCSHL-338), which was over expressed during hypoxic condition was also characterized. HSP40, HSP70 and HSP90 molecules are known chaperonins acting as molecular chaperones. These work in tandem to assist maturation of newly synthesized proteins and prevent aggregation of proteins when cells are subjected to various forms of stress (Wang, Vinocur, Shoseyov, & Altman, 2004a; Lanneau, de Thonel, Maurel, Didelot, & Garrido, 2007). HIF-1, a transcription factor, is involved in the metabolic switch to anaerobic glycolysis (Soitamo, Rabergh, Gassmann, Sistonen, & Nikinmaa, 2001; Trisciuoglio *et al*., 2010). HSP90 was reported to be a major regulator of HIF-1 activation (Minet *et al*., 1999). Prefoldin, a ubiquitously expressed heterohexameric co-chaperone, is necessary for proper folding of nascent proteins, in particular, tubulin and actin filaments. Prefoldin is also a molecular chaperone that mediates transfer of newly synthesized proteins from HSP complexes to the cytosolic chaperonin (Young, Agashe, Siegers, & Hartl, 2004). In *Arabidopsis*, Prefoldins 3 and 5 mediated proper cytoskeleton formation during salt stress tolerance (Rodriguez-Milla & Salinas, 2009). Here, we report that prefoldin 6 transcript expression is up-regulated. It is likely to be involved in hypoxia stress mitigation. During prolonged hypoxia, possibly the TCA (Tricarboxylic acidic acid cycle) cycle was lifted and the response was shifted towards up-regulation of gene encoding fatty acid-binding protein. Fatty acid-binding protein containing lipocalin domain are likely to be associated with hypoxic stress management. It was observed that lipocalin 2 is expressed in cortical neurons and could potentially be involved in apoptotic pathways following hypoxia. Its potentiating activity during hypoxia was also documented earlier (Ralph *et al*., 2004).

Earlier studies suggested that hypoxia stimulates the expression of uPA receptor domain (Graham, Fitzpatrick, & McCrae, 1998; Noh, Hong, & Huang, 2013). In line with this, we have characterized an uPA domain containing new cDNA (CSHL-338, uncharacterized) that contains 5′- and 3′- UTRs. The long 5′-UTR of CSHL-338 indicated about its possible involvement in regulating expressions linked to adaptive response to a particular environmental situation.

The transcript level data presented in this study validated that hypoxia treatment to *C. striatus* was successfully applied in experimental conditions that led to either reduced metabolic rate to match the reduced supply of energy or maintain metabolic rate by increasing anaerobic metabolism (glycolysis) so as to adjust the ATP demand. Evidences are provided with regard to wide-scale changes in gene expressions linked to series of adaptive responses against hypoxic stress. Several defense mechanisms such as a drastic suppression of ATP demand inclusive of shutting-down costly energy processes (such as protein synthesis, cell division and ion pumping activities) were operative. The over expressed transcripts with no strong BLAST homology amongst genes induced by hypoxia identified in this study could be considered as novel ones and those are most likely to be associated with hypoxia tolerance. It could be argued that behavioral changes, particularly hibernation of *C. striatus* during O_2 deficiency, could well be associated with a phenomenon of estivation rather than hypoxia. However, estivation is linked to hypoxia (Brooks & Storey, 1990; Whitwam & Storey, 1990), where physiological adaptive mechanisms of hypoxia and estivation share several common features (Giusi *et al*., 2012).

In this study, differential expression patterns have been averaged among the four time points of 39, 45, 47 and 61 days treatments. It is essential to elucidate the exact sequential *in vivo* events operative during hypoxia adaptation. However, the aim of the study was to identify overall changes in gene expression patterns during long term hypoxia exposure. The differential gene expression at different time points could be undertaken in future studies. The transcript levels are only a proxy for protein expressions, and may not be identical completely with protein expression because of post-translational modifications or other reasons. Future studies should be undertaken to confirm transcriptome results with proteome. Nevertheless, changes in mRNA expression patterns, as observed in this study in hypoxic snakehead fish, could well be utilized as molecular indicators for detecting exposure to prolonged hypoxia.

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References

Abdulmalek, K., Ashur, F., Ezer, N., Ye, F., Magder, S., & Hussain, S.N. (2001). Differential expression of Tie-2 receptors and angiopoietins in response to *in vivo* hypoxia in rats. *American Journal of Physiology: Lung Cellular and Molecular Physiology*, 281(3), L582-590. http://ajplung.physiology.org/

Almgren, C.M., & Olson, L.E. (1999). Moderate hypoxia increases heat shock protein 90 expression in excised rat aorta. *Journal of Vascular Research*, 36(5), 363-371. 25675. http://dx.doi:10.1159/000025675

Altschul, S., Gish, W., Miller, W., Myers, E., & Lipman, D. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215(3), 403-410. http://dx.doi:10.1016/S0022-2836(05)80360-2

Aly, H., Hamed, Z., Mohsen, L., Ramy, N., Arnaoot, H., & Lotfy, A. (2011). Serum amyloid A protein and hypoxic ischemic encephalopathy in the newborn. *Journal of Perinatology*, 31(4), 263-268. http://dx.doi:10.1038/jp.2010.130

Barman, H.K., Mohanta, R., Patra, S.K., Chakrapani, V., Panda, R.P., Nayak, S., . . . Nandanpawar, P. (2015). The β-actin gene promoter of rohu carp (*Labeo rohita*) drives reporter gene expressions in transgenic rohu and various cell lines, including spermatogonial stem cells. *Cell Molecular Biology Letters*, 20(2), 237-247. http://dx.doi:10.1515/cmble-2015-0010

Barman, H.K., Panda, R.P., Mohapatra, C., Swain, A., & Eknath, A.E. (2011). Identification of genes preferentially expressed in testis and spermatogonial cells of *Labeo rohita* by subtractive and suppressive hybridization. *Aquaculture Research*, 42(8), 1196-1205. http://dx.doi:10.1111/j.1365-2109.2010.02710

Barman, H.K., Patra, S.K., Das, V., Mohapatra, S.D., Jayasankar, P., Mohapatra, C., . . . Rath, S.N. (2012). Identification and characterization of differentially expressed transcripts in the gills of freshwater prawn (*Macrobrachium rosenbergii*) under salt stress. *The Scientific World Journal*, 2012, 149361. http://dx.doi:10.1100/2012/149361

Bauer, E.M., Zheng, H., Comhair, S., Erzurum, S., Billiar, T.R., & Bauer, P.M. (2011). Complement C3 deficiency attenuates chronic hypoxia-induced pulmonary hypertension in mice. *PLoS One*, 6(12), e28578. http://dx.doi: 10.1371/journal.pone.0028578

Baze, M.M., Schlauch, K., & Hayes, J.P. (2010). Gene expression of the liver in response to chronic hypoxia. *Physiological Genomics*, 41(3), 275-288. http://dx.doi: 10.1152/physiolgenomics.00075.2009

Bernier, N., Harris, J., Lessard, J., & Randall, D. (1996). Adenosine receptor blockade and hypoxia-tolerance in rainbow trout and Pacific hagfish. I. Effects on anaerobic metabolism. *Journal of Experimental Biology*, 199(2), 485-495. Ritrieved from http://jeb.biologists.org/content/jexbio/199/2/485

Brooks, S.P.J., & Storey, K.B. (1990). Glycolytic enzyme binding and metabolic control in estivation and anoxia in the land snail *Otala Lactea*, *Journal of Experimental Biology*, 151, 193-204. Retrieved from http://jeb.biologists.org/

Brouwer, M., Brown-Peterson, N.J., Hoexum-Brouwer, T., Manning, S., & Denslow, N. (2008). Changes in mitochondrial gene and protein expression in grass shrimp, *Palaemonetes pugio*, exposed to chronic hypoxia. *Marine Environmental Research*, 66(1), 143-145. http://dx.doi:10.1016/j.marenvres.2008.02.046

Brown-Peterson, N.J., Larkin, P., Denslow, N., King, C., Manning, S., & Brouwer, M. (2005). Molecular indicators of hypoxia in the blue crab *Callinectes sapidus*. *Marine Ecology Progress Series*, 286, 203-215. http://www.int-res.com/articles/meps2005/286/

m286p203

Chakrapani, V., Patra, S.K., Panda, R.P., Rasal, K.D., Jayasankar, P., & Barman, H.K. (2016). Establishing targeted carp TLR22 gene disruption via homologous recombination using CRISPR/Cas9. *Developmental & Comparative Immunology*, 61, 242-247. http://dx.doi:10.1016/j.dci.2016.04.009

Chandra, S., & Banerjee, T. (2004). Histopathological analysis of the respiratory organs of *Channa striatus* subjected to air exposure. *Veterinarski Archiv*, 74(1), 37-52. http://www-staro.vef.unizg.hr/vetarhiv/

Crans, K.D., Pranckevicius, N.A., & Scott, G.R. (2015). Physiological tradeoffs may underlie the evolution of hypoxia tolerance and exercise performance in sunfish (Centrarchidae). *The Journal of Experimental Biology*, 218(20), 3264-3275. http://dx.doi:10.1242/jeb.124602

David, E., Tanguy, A., Pichavant, K., & Moraga, D. (2005). Response of the Pacific oyster *Crassostrea gigas* to hypoxia exposure under experimental conditions. *Federation of European Biochemical Societies Journal*, 272(21), 5635-5652. http://dx.doi:10.1111/j.1742-4658.2005.04960.x

Davidson, J.F., & Schiestl, R.H. (2001). Mitochondrial respiratory electron carriers are involved in oxidative stress during heat stress in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*, 21(24), 8483-8489. http://dx.doi:10.1128/MCB.21.24.8483-8489.2001

Diatchenko, L., Lukyanov, S., Lau, Y.F., & Siebert, P.D. (1999). Suppression subtractive hybridization: a versatile method for identifying differentially expressed genes. *Methods in Enzymology*, 303, 349-380. http://dx.doi: 10.1016/S0076-6879(99)03022-0

Fan, Q.J., Yan, F.X., Qiao, G., Zhang, B.X., & Wen, X.P. (2014). Identification of differentially-expressed genes potentially implicated in drought response in pitaya (*Hylocereus undatus*) by suppression subtractive hybridization and cDNA microarray analysis. *Gene*, 533(1), 322-331. http://dx.doi:10.1016/j.gene.2013.08.098

Flight, P.A., Nacci, D., Champlin, D., Whitehead, A., & Rand, D.M. (2011). The effects of mitochondrial genotype on hypoxic survival and gene expression in a hybrid population of the killifish, *Fundulus heteroclitus*. *Molecular Ecology*, 20(21), 4503-4520. http://dx.doi:10.1111/j.1365-294X.2011.05290.x

Fraser, J., de Mello, L.V., Ward, D., Rees, H.H., Williams, D.R., Fang, Y., ... Cossins, A.R. (2006). Hypoxia-inducible myoglobin expression in nonmuscle tissues. *Proceedings of National Acadamy of Sciences*, 103(8), 2977-2981. http://dx.doi:10.1073/pnas.0508270103

Fu, X., Huang, Y., Deng, S., Zhou, R., Yang, G., Ni, X., ... Shi, S. (2005). Construction of a SSH library of *Aegiceras corniculatum* under salt stress and expression analysis of four transcripts. *Plant Science*, 169(1), 147-154. http://dx.doi:10.1016/j.plantsci.2005. 03.009

Gamperl, A.K., Faust, H.A., Dougher, B., & Rodnick, K.J. (2004). Hypoxia tolerance and preconditioning are not additive in the trout (*Oncorhynchus mykiss*) heart. *Jounal of Experimental Biology*, 207, 2497-2505. http://dx.doi:10.1242/jeb.01055

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R., & Bairoch, A. (2003). ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Research*, 31(13), 3784-3788. http://dx.doi:10.1093/nar/gkg563

Giusi, G., Zizza, M., Facciolo, R.M., Chew, S.F., Ip, Y.K., & Canonaco, M. (2012). Aestivation and hypoxia-related events share common silent neuron trafficking processes. *BMC Neurosciences*, 13(39). http://dx.doi:10.1186/1471-2202-13-39

Goswami, S., Kumar, R.R., Dubey, K., Singh, J.P., Tiwari, S., Kumar, A., . . . Rai, R.D. (2016). SSH analysis of endosperm transcripts and characterization of heat stress regulated Expressed Sequence Tags in Bread Wheat. *Frontiers in Plant Science*, 17(7), 1230. http://dx.doi:10.3389/fpls.2016.01230

Gracey, A.Y., Troll, J.V., & Somero, G.N. (2001). Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. *Proceedings of National Acadamy of Sciences*, 98(4), 1993-1998. http://dx.doi:10.1073/pnas.98.4.1993 98/4/1993

Graham, C.H., Fitzpatrick, T.E., & McCrae, K.R. (1998). Hypoxia stimulates urokinase receptor expression through a heme protein-dependent pathway. *Blood*, 91(9), 3300-3307. Retrieved from http://www.bloodjournal.org/content/bloodjournal/91/9/3300

Graham, J. (1997). Air-breathing fishes: evolution, diversity and apatation. New York, USA, Academic Press., 58 pp.

Gunther, A.C.L.G. (1880). An introduction to the study of fishes. New Delhi, India, Today and Tomorrow's Book Agency., 513 pp.

Heads, R.J., Yellon, D.M., & Latchman, D.S. (1995). Differential cytoprotection against heat stress or hypoxia following expression of specific stress protein genes in myogenic cells. *Journal of Molecular and Cellular Cardiology*, 27(8), 1669-1678. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/8523429

Hochachka, P.W., & Lutz, P.L. (2001). Mechanism, origin, and evolution of anoxia tolerance in animals. *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology*, 130(4), 435-459. http://dx.doi.org/10.1016/S1096-4959(01)00408-0

Kozak, M. (1991). Structural features in eukaryotic mRNAs that modulate the initiation of translation. *The Journal of Biogical Chemistry*, 266(30), 19867-19870. http://www.jbc.org/content/266/30/19867

Lanneau, D., de Thonel, A., Maurel, S., Didelot, C. & Garrido, C. (2007). Apoptosis versus cell differentiation: role of heat shock proteins HSP90, HSP70 and HSP27. *Prion*, 1(1), 53-60. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2633709/pdf/prion0101_0053

Leveelahti, L., Leskinen, P., Leder, E.H., Waser, W., & Nikinmaa, M. (2011). Responses of threespine stickleback (*Gasterosteus aculeatus*, L) transcriptome to hypoxia. C*omparative Biochemistry and Physiology Part D Genomics Proteomics*, 6(4), 370-381. http://dx.doi:10.1016/j.cbd.2011.08.001

Marchler-Bauer, A., & Bryant, S. (2004). CD-Search: protein domain annotations on the fly. *Nucleic Acids Research*, 32, 327-331. http://dx.doi: 10.1093/nar/gkh454

Marques, I.J., Leito, J.T., Spaink, H.P., Testerink, J., Jaspers, R.T., Witte, F., ... Bagowski, C.P. (2008). Transcriptome analysis of the response to chronic constant hypoxia in zebrafish hearts. *Journal Comparative Physiology B*, 178(1), 77-92. http://dx.doi:10.1007/s00360-007-0201-4

Martinovic, D., Villeneuve, D.L., Kahl, M.D., Blake, L.S.,

Brodin, J.D., & Ankley, G.T. (2009). Hypoxia alters gene expression in the gonads of zebrafish (*Danio rerio*). *Aquatic Toxicology*, 95(4), 258-272. http://dx.doi:10.1016/j.aquatox.2008.08.021

McCurley, A.T., & Callard, G.V. (2008). Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. *BMC Molecular Biology*, 9(102). http://dx.doi:10.1186/1471-2199-9-102

Mestdagh, P., Van Vlierberghe, P., De Weer, A., Muth, D., Westermann, F., Speleman, F., & Vandesompele, J. (2009). A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biology*, 10(6), R64. http://dx.doi:10.1186/gb-2009-10-6-r64

Minet, E., Mottet, D., Michel, G., Roland, I., Raes, M., Remacle, J., & Michiels, C. (1999). Hypoxia-induced activation of HIF-1: role of HIF-1alpha-Hsp90 interaction. *FEBS Letters*, 460(2), 251-256. http://dx.doi: 10.1016/S0014-5793(99)01359-9

Mohanta, R., Jayasankar, P., Mahapatra, K.D., Saha, J.N., & Barman, H.K. (2014). Molecular cloning, characterization and functional assessment of the myosin light polypeptide chain 2 (*myl*z2) promoter of farmed carp, *Labeo rohita*. *Transgenic Research*, 23(4), 601-607. http://dx.doi:10.1007/s11248-014-9798-8

Mohapatra, C., & Barman, H.K. (2014). Identification of promoter within the first intron of *Plzf* gene expressed in carp spermatogonial stem cells. *Molecular Biolog Report*, 41(10), 6433-6440. http://dx.doi:10.1007/s11033-014-3525-7

Mohapatra, C., Barman, H.K., Panda, R.P., Kumar, S., Das, V., Mohanta, R., . . . Jayasankar, P. (2010). Cloning of cDNA and prediction of peptide structure of *Plzf* expressed in the spermatogonial cells of *Labeo rohita*. *Marine Genomics*, 3(3-4), 157-163. http://dx.doi:10.1016/j.margen.2010.09.002.

Mohapatra, C., Patra, S.K., Panda, R.P., Mohanta, R., Saha, A., Saha, J.N., . . . Barman, H.K. (2014). Gene structure and identification of minimal promoter of *Pou*2 expressed in spermatogonial cells of rohu carp, *Labeo rohita*. *Molecular Biology Report*, 41(6), 4123-4132. http://dx.doi:10.1007/s11033-014-3283-6

Mohapatra, S.D., Kumar, K., Jayasankar, P., & Barman, H.K. (2013). Establishment of dry-down hypoxic stress treatment protocol for snakehead freshwater fish, *Channa striatus*. *International Journal of Fisheries and Aquatic Studies*, 1(2), 36-39. http://www.fisheriesjournal.com/vol1issue2/pdf/10.1.

Morey, J., Ryan, J., & Van Dolah, F. (2006). Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. *Biological Procedure Online*, 8, 175-193. http://dx.doi:10.1251/bpo126

Mottet, D., Ruys, S.P., Demazy, C., Raes, M., & Michiels, C. (2005). Role for casein kinase 2 in the regulation of HIF-1 activity. *International Journal of Cancer*, 117(5), 764-774. http://dx.doi:10.1002/ijc.21268

Noh, H., Hong, S., & Huang, S. (2013). Role of urokinase receptor in tumor progression and development. *Theranostics*, 3(7), 487-495. http://dx.doi:10.7150/thno.4218thnov03p0487

Overgaard, J., Stecyk, J.A., Gesser, H., Wang, T., Gamperl, A.K., & Farrell, A.P. (2004). Preconditioning stimuli do not benefit the myocardium of hypoxia-tolerant rainbow trout (*Oncorhynchus mykiss*). *Journal of Comparative Physiology B*, 174(4), 329-340. http://dx.doi:10.1007/s00360-004-0418-4

Panda, R.P., Barman, H.K., & Mohapatra, C. (2011). Isolation of enriched carp spermatogonial stem cells from *Labeo rohita* testis for *in vitro* propagation. *Theriogenology*, 76(2), 241-251. http://dx.doi:10.1016/j.theriogenology.2011.01.031

Panda, R.P., Chakrapani, V., Patra, S.K., Saha, J.N., Jayasankar, P., Kar, B., ... Barman, H.K. (2014). First evidence of comparative responses of Toll-like receptor 22 (TLR22) to relatively resistant and susceptible Indian farmed carps to *Argulus siamensis* infection. *Devlopmental & Comparative Immunology*, 47(1), 25-35. http://dx.doi:10.1016/j.dci.2014.06.016

Patra, S.K., Chakrapani, V., Panda, R.P., Mohapatra, C., Jayasankar, P., & Barman, H.K. (2015). First evidence of molecular characterization of rohu carp *Sox*2 gene being expressed in proliferating spermatogonial cells. *Theriogenology*, 84(2), 268-276. http://dx.doi:10.1016/j.theriogenology.2015.03.017

Rajaraman, G., Wang, G.Q., Yan, J., Jiang, P., Gong, Y., & Burczynski, F.J. (2007). Role of cytosolic liver fatty acid binding protein in hepatocellular oxidative stress: effect of dexamethasone and clofibrate treatment. *Molecular and Cellular Biochemistry*, 295(1-2), 27-34. http://dx.doi:10.1007/s11010-006-9268-6

Ralph, G.S., Parham, S., Lee, S.R., Beard, G.L., Craigon, M.H., Ward, N., ... Krige, D. (2004). Identification of potential stroke targets by lentiviral vector mediated overexpression of HIF-1 alpha and HIF-2 alpha in a primary neuronal model of hypoxia. *Journal of Cerebral Blood Flow & Metabolism*, 24(2), 245-258. http://dx.doi:10.1097/01.WCB.0000110532.48786.46

Richards, J.G., Farrell, A.P., & Brauner, C.J. (2009). Fish Physiology: Hypoxia. Burlington, United States, Academic Press., 528 pp.

Rodriguez-Milla, M.A., & Salinas, J. (2009). Prefoldins 3 and 5 play an essential role in Arabidopsis tolerance to salt stress. *Molecular Plant*, 2(3), 526-534. http://dx.doi:10.1093/mp/ssp016

Rolfs, A., Kvietikova, I., Gassmann, M., & Wenger, R.H. (1997). Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. *The Journal of Biological Chemistry*. 272(32), 20055-20062. http://dx.doi:10.1074/jbc.272.32.20055August 8, 1997

Semenza, G.L. (2007). Oxygen-dependent regulation of mitochondrial respiration by hypoxia-inducible factor 1. *Biochemical Journal*, 405(1), 1-9. http://dx.doi: 10.1042/BJ20070389

Semenza, G.L., Jiang, B.H., Leung, S.W., Passantino, R., Concordet, J.P., Maire, P., & Giallongo, A. (1996). Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *The Journal of Biological Chemistry*, 271(51), 32529-32537. http://dx.doi:10.1074/jbc.271.51.32529

Shen, C., Nettleton, D., Jiang, M., Kim, S.K., & Powell-Coffman, J.A. (2005). Roles of the HIF-1 hypoxia-inducible factor during hypoxia response in *Caenorhabditis elegans*. *The Journal of Biological Chemistry*, 280(21), 20580-20588. http://dx.doi:10.1074/jbc.M501894200

Soitamo, A.J., Rabergh, C.M., Gassmann, M., Sistonen, L., & Nikinmaa, M. (2001). Characterization of a hypoxia-inducible factor (HIF-1alpha) from rainbow trout. Accumulation of protein occurs at normal

venous oxygen tension. *The Journal of Biological Chemistry*, 276(23), 19699-19705. http://dx.doi:10.1074/jbc.M009057200

Storey, K.B. (2004). Molecular mechanisms of anoxia tolerance. *International Congress Series*, 1275, 47-54. http://dx.doi:10.1016/j.ics.2004.08.072

Storey, K.B. (2007). Anoxia tolerance in turtles: metabolic regulation and gene expression. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 147(2), 263-276. http://dx.doi:10.1016/j.cbpa.2006.03.019

Sugimoto, T., Mori, C., Takanami, T., Sasagawa, Y., Saito, R., Ichiishi, E., & Higashitani, A. (2008). *Caenorhabditis elegans* par2.1/mtssb-1 is essential for mitochondrial DNA replication and its defect causes comprehensive transcriptional alterations including a hypoxia response. *Experimental Cell Research*, 314(1), 103-114. http://dx.doi:10.1016/j.yexcr.2007.08.015

Sussarellu, R., Fabioux, C., Le Moullac, G., Fleury, E., & Moraga, D. (2010). Transcriptomic response of the Pacific oyster *Crassostrea gigas* to hypoxia. *Marine Genomics*, 3(3-4), 133-143. http://dx.doi:10.1016/j.margen.2010.08.005

Tacchini, L., Bianchi, L., Bernelli-Zazzera, A., & Cairo, G. (1999). Transferrin receptor induction by hypoxia. HIF-1-mediated transcriptional activation and cell-specific post-transcriptional regulation. *The Journal of Biological Chemistry*, 274(34), 24142-24146. Retrieved from http://www.jbc.org/

Tian, B., Hu, J., Zhang, H., & Lutz, C.S. (2005). A large-scale analysis of mRNA polyadenylation of human and mouse genes. *Nucleic Acids Res*earch, 33(1), 201-212. http://dx.doi:10.1093/nar/gki158

Tohgi, H., Utsugisawa, K., & Nagane, Y. (2000). Hypoxia-induced expression of C1q, a subcomponent of the complement system, in cultured rat PC12 cells. *Neuroscience Letters*, 291(3), 151-154. http://dx.doi: 10.1016/S0304-3940(00)01399-9

Ton, C., Stamatiou, D., & Liew, C.C. (2003). Gene expression profile of zebrafish exposed to hypoxia during development. *Physiological Genomics*, 13(2), 97-106. http://dx.doi:10.1152/physiolgenomics.00128.2002

Trisciuoglio, D., Gabellini, C., Desideri, M., Ziparo, E., Zupi, G., & Del Bufalo, D. (2010). Bcl-2 regulates HIF-1alpha protein stabilization in hypoxic melanoma cells via the molecular chaperone HSP90. *PLoS One*, 5(7), e11772. http://dx.doi:10.1371/journal.pone. 0011772

UniProt, C. (2011). Ongoing and future developments at the Universal Protein Resource. *Nucleic Acids Research*, 39, D214-D219. http://dx.doi:10.1093/nar/gkq1020

Wang, G., Gong, Y., Anderson, J., Sun, D., Minuk, G., Roberts, M.S., & Burczynski, F.J. (2005). Antioxidative function of L-FABP in L-FABP stably transfected Chang liver cells. *Hepatology*, 42(4), 871-879. http://dx.doi: 10.1002/hep.20857

Wang, W., Vinocur, B., Shoseyov, O., & Altman, A. (2004a). Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends in Plant Science*, 9(5), 244-252. http://dx.doi:10.1016/j.tplants.2004.03.006

Wang, X., Deng, J., Boyle, D.W., Zhong, J., & Lee, W.H. (2004b). Potential role of IGF-I in hypoxia tolerance using a rat hypoxic-ischemic model: activation of hypoxia-inducible factor 1alpha. *Pediatric Research*, 55(3),385-394. http://dx.doi:10.1203/01.pdr.0000111482.43827.4001

Whitwam, R.E., & Storey, K.B. (1990). Pyruvate kinase from the land snail *Otala Lactea*: regulation by reversible phosphorylation during estivation and anoxia. *Journal of Experimental Biology*, 154, 321-337. Retrieved from http://kenstoreylab.com/wp-content/uploads/2016/03/

Woo, S., Jeon, H.Y., Kim, S.R., & Yum, S. (2011). Differentially displayed genes with oxygen depletion stress and transcriptional responses in the marine mussel, *Mytilus galloprovincialis*. *Comparative Biochemistry and Physiology Part D Genomics Proteomics*, 6(4), 348-356. http://dx.doi:10.1016/ j.cbd.2011.07.003

Young, J.C., Agashe, V.R., Siegers, K., & Hartl, F.U. (2004). Pathways of chaperone-mediated protein folding in the cytosol. *Nature Reviews Molecular Cell Biology*, 5(10), 781-791. http://dx.doi:10.1038 /nrm1492

Zhao, P.J., Pan, J., Li, F., & Sun, K. (2008). Effects of chronic hypoxia on the expression of calmodulin and calcicum/calmodulin-dependent protein kinase II and the calcium activity in myocardial cells in young rats. *Chinese journal of contemporary pediatrics*, 10(3), 381-385. https://www.ncbi.nlm.nih.gov/pubmed/ 18554473