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ARTICLE

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EXPRESSION KINETIC STUDY OF HSP70 GENE ASSOCIATED TRANSPORTATION STRESS OF *TENUALOSA ILISHA* (HAMILTON 1822)

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The Farakka Barrage has caused a decline in Hilsa (Tenualosa ilisha) populations in the Ganga River's upper stretch. To restore the population, a ranching program was launched in 2019. This study evaluates Hsp70 gene expression during transportation, highlighting its importance as a stress assessment and survival improvement biomarker. Results showed weak Hsp70 expression in control fish (ratio 0.85), while transported fish exhibited ratios between 1.16 and 3.6. Stress increased significantly after 5 km of transport, with RNA quality ranging from 1.20 ± 0.11 to 1.80 ± 0.11 µg/mg and a decline in RNA: DNA ratio, indicating heightened stress levels. The study found that the Hsp70 gene's expression varied depending on distance. Understanding these factors can help develop new protocols to reduce stress and increase survival rates.

Keywords: *Biological Factors, Biomarkers, Heat Shock Protein, Stress, Transportation*

Introduction

Usually, stress is caused in fish during transportation processes. This study investigated the effect of transport stress on the hepatic heat shock protein (HSPs) of *Tenualosa ilisha.* Transport of live fish is an unavoidable procedure in the aquaculture system. The transport process includes several procedures whether pre-transport procedures (such as collection, grading, netting, air exposure, and packing) and during transport process procedures (such as water movement, vibrations, and water condition change), which are stressful to fish[1-3]. The stress resulting from transport often causes fish growth suppression, high mortality[4], susceptibility to diseases[5], and the increment of rearing costs[6]. Consequently, the Transport procedure should be designed to minimize stress[7].

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On the cellular level, stress stimulates a physiological response through increased synthesis of heat shock proteins (HSPs). HSPs include a large group of conserved proteins, classified according to their molecular weight[8]. HSPs regulate cellular protein structure and act as housekeeping and cytoprotective function[9], as well as, it works as a companion to the metabolic activities of both protein and lipid[10]. Additionally HSPs play an important role in cellular homeostasis under abrupt environmental fluctuation[11]. HSPs Families include Hsp70 and Hsp70, which work on the folding and aggregation of cellular proteins and regulation of kinetic partitioning between folding, translocation, and assemblage within the cell[10]. The mRNA expression of HSPs has been studied in many fish species exposed to various stress conditions such as temperature extremes, pollutants, confinement, handling stress, and acclimatization to salinities[12-15]. Consequently, Poltronieri et al.,[16] suggested that Hsp70 could be used for evaluating welfare condition in fish. However, studies on the effect of transport stress on HSPs are limited in Hilsa.

Table 1 Primer used in this study

Gene	Orientation	Primer	Len	Tm(°c)	Product size
Hsp70	FORWARD	GGCACCACCTACTCCTGTGT	20	58-60	688bp
	REVERSE	AGTGACTAACCAGGCGGTTG	20	58-60	
β-actin	FORWARD	TTCGAGCAGGAGATGGGCACTG	22	55-65	254bp
	REVERSE	GCATCCTGTCAGCAATGCCA	20	55-65	

Nowadays, Hilsa has become one of the most popular species in India. However, no study has reported changes in the defence system and hepatic HSPs mRNA expression in Hilsa suffering from transport stress. Hence, this study aimed to investigate the effect of transport on HSPs mRNA expression. This study will assist people in understanding the physiological responses of Hilsa suffering from transport stress and improve fish welfare.

Materials and Methods

Ethical Statement: This work was approved by the ICAR-Central Inland Fisheries Research Institute (ICAR-CIFRI) Animal Ethics Committee. All of the fish used in the experiment were handled according to the Institute's prescribed guidelines.

***Experimental Fish and Tissue Collection*:** During the transport of the adult Hilsa fish (100-200 g) an average length of 15.98±1.52 from downstream to upstream of the Farakka barrage (Lat. 20 ° 1'06 "- 20 ° 11.45" N long, 80 ° 50'52 " - 85 ° 51'35``E) for the ranching program. Fish were subjected to netting, handling, and grading before the transport process, and thereafter split into two groups. One was the control group (the non-transported fish) which were kept in normal condition and at a density of three fish per tank (330 L). Another group of fish was exposed to the transport stress (the treatment group) The sample was collected during transportation at different distance intervals (i.e., 0 km, 1 km, 2 km, 3 km, 4 km, 5 km, 6 km, 7 km, and 10 km) and euthanized with MS-222 immediately before dissection. Brain samples were collected from each fish, quickly stored in RNA later, and kept in the icebox for the RNA extraction.

RNA Isolation and Reverse Transcriptase: Following the guidelines provided by the manufacturer, total RNA was extracted from samples of 50 to 100 mg of brain tissue using the Trizol-reagent (GCC Biotech, West Bengal); the RNA pellet was washed in 75% ethanol twice and finally suspended in 50µl sterile di-ethylpyrocarbonate-treated water. RNA's optimal density was determined to be 260/280 nm using nanodrop (BioTek, EPOCH Micro-plate Reader). The qualitative expression of 28 and 18S ribosomal RNA on 1% agarose gel was used to assess the integrity of the extracted RNA. RNA was reverse transcribed into cDNA with Oligo-dT priming For the first-strand DNA synthesis. Five micrograms of total RNA were processed using one unit of DNase I (Sigma, USA). The DNase-treated RNA was mixed with 0.33 µg of Random primer to a 10 µl volume, heated to 65°C for 10 min, and then immediately chilled on ice. Using ABI Biosystems' Reverse transcription was carried out by taking 1 µl of 100 unit multiscribe reverse transcriptase enzyme, 0.8 µl of 100 mM dNTP, RT buffer (25 mM Tris-HCL pH 8.3, 37.5 mM KCL,1.5 mM MgCl2, 10 mM DTT) and RNase inhibitor were used to perform reverse transcription. Samples were adjusted to a final volume of 20 µl and incubated for 60 min at 37°C. The cDNA was stored at -20 °C for future use.

DNA and RNA/DNA Ratio: DNA was extracted using a DNA isolation kit (Qiagen) according to the

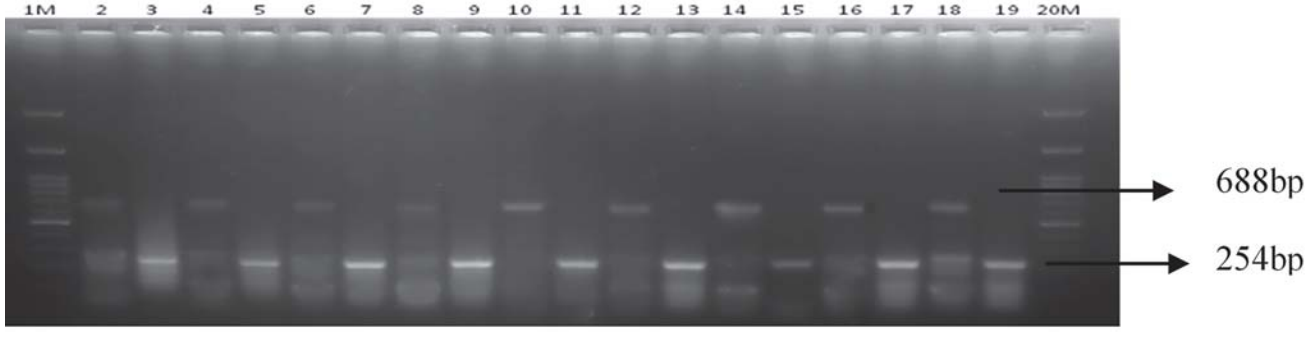

Fig. 1: HSP70 and β-actin expression in the Hilsa brain tissues
L1–100bp Marker, L5 β-actin-1h, L9 β-actin-3h, L13 β-actin-5h L17 β-actin-7h
L2–Hsp70-0h, L6 Hsp70-2h, L10 Hsp70-4h, L14 Hsp70-6h, L18 Hsp70-10h
L3–β-actin-0h, L7 β-actin-2h, L11 β-actin-4h, L15 β-actin-6h, L19 β-actin-10h
L4–Hsp70-1h, L8 Hsp70-3h, L12 Hsp70-5h, L16 Hsp70-7h, L20 100bp Marker

manufacturer's instructions. The genomic DNA concentration was calculated Using Nanodrop (BioTek, EPOCH Micro-plate Reader). The RNA / DNA ratio was calculated after quantification.

Primer Design and Hsp70 Amplification: The primer was designed from the known sequences of the Hsp70 gene available in the gene bank, and the primer was designed using fast PCR software. To amplify the partial cDNA sequence of the hilsa Hsp70 gene, PCR was done with primers (F-5'-GGCACCACCTACTCCTGTGT -3' and R-5'- AGTGACTAACCAGGCGGTTG-3') designed from the conserved region of previously reported fish Hsp70 cDNA sequences. The β-actin and Hsp70 PCR results were run in adjacent lanes on a 1.5% agarose gel and stained with ethidium bromide to make them visible. To counteract the variation in RNA quality and reverse transcription efficiency between individuals, the intensity of Hsp70 signals was measured by image analysis (Gene Genius Bio Imaging System, SynGene) and divided by the intensity of the respective β-actin signals to give a ratio of Hsp70: β -actin signal strength for each sample.

q PCR Analysis: Internal PCR was used to amplify part of the housekeeping gene encoding β-actin. Semi-quantitative analysis of the Hsp70 gene and the reference gene β-actin was performed in a thermal cycler (Applied Biosystems, USA). The cycle conditions were optimized for both the Hsp70 and the β-actin housekeeping genes. Primers specific for Hsp70 (forward 5'-GGCACCACCTACTCCTGTGT -3 'and reverse 5'-AGTGACTAACCAGGCGGTTG -3') and β-actin (forward, 5'TTCGAGCAGGAGATGGGCACTG 3'; reverse, 5' GCATCCTGTCAGCAATGCCA 3') were used to amplify fragments of 700 and 254 bp of Hsp70 and β-actin, respectively. The amplification was carried out in 25 µl reaction volume is carried out, which is 2 µl cDNA, 1 µl each of forward and reverse primers (10 pM), 0.2 µl dNTP (100 mM, Sigma), 3.5 µl $MgCl_2$ (100 mM, Sigma), 2.5 µl 10x reaction buffer (Sigma), 1 unit of Taq DNA polymerase (5 U / µl, Sigma). The PCR amplification was performed under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C / 30 s, 55°C for 45 s, and 72°C for 45 s, and final extension at 72°C for 7 min. To confirm the correct size and single band amplification, 8 µl of the qRT-PCR products were analyzed by electrophoresis in ethidium bromide-stained 1.2% agarose gel and visualized in a gel-doc system.

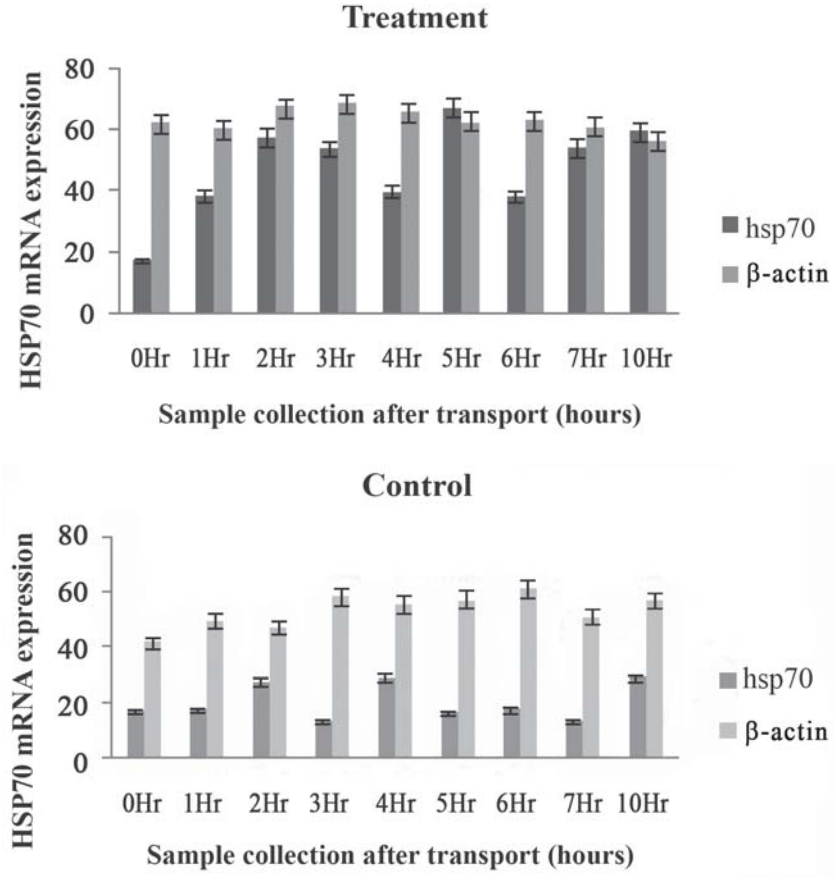

Fig. 2a, b: Hsp70 mRNA Expression with compare β-actin in Hilsa, during transportation

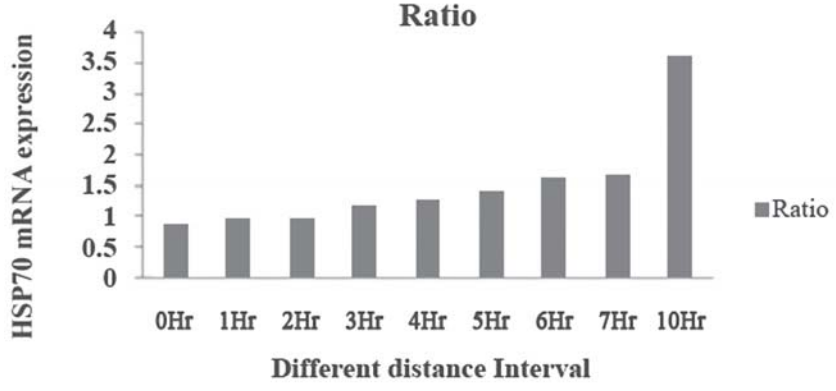

Fig. 3: Hsp70 ratio showing Hsp70 expression at different distance interval during transport.

Results

Hsp70 Expression in Different Brain Tissue with Respect to Different Distance Interval: Control fish (0 km) show weak Hsp70 expression (ratio 0.85), while on the other hand, a mean value of the Hsp70 and β-actin ratio of 1.16-3.6 is present for different distance intervals. After 5 km of transport, the stress label was very high. (Figure. 1, 2 and 3).

RNA, DNA and RNA/DNA Ratio: The DNA quality did not vary significantly between the different groups. However, the RNA quality varied significantly in the range of 1.20 ± 0.11 µg / mg to 1.80 ± 0.11 µg / mg. The RNA: DNA ratio decreased significantly due to the high labelling stress.

Discussion

The potential application of Hsp as biomarkers continues to be an important subject of research in environmental physiology. Their universality, extraordinary conservation in structure, and the consistency with which a broad spectrum of stressors induces them have made HSPs good candidates for biomonitoring of the environment. At molecular levels, the increased Hsp70 expression may be considered as indicative of cellular alteration, and due to its highly inducible nature, this protein may act as an excellent mediator of cellular stress[17]. In our experiment, during the transport of Hilsa fish, the Hsp70 gene stimulates and expresses different trends depending on the distance and is unregulated continuously. Therefore, this expression would be modulated by multiple environmental and biological factors, which does not diminish the importance of evaluating the Hsp70 as a stress biomarker.

Conclusion

During the transport of Hilsa fish, the Hsp70 gene stimulates and expresses different trends depending on the distance. This can be due to a lack of oxygen or modulation of disruption of ionic balance and cellular responses to stress are expected to be activated. This is an area that needs more attention from researchers to standardize the transportation of hilsa fish for the ranching program.

Acknowledgments

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