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Fish Stress Protein: An Approach for Biomonitoring of Water Quality in Bangladesh

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Abstract Need exists for rapid means of assessing the "health" of water bodies in Bangladesh. This study aims to assess health indicator for aquatic lives. Response to environmental stress extends to tissue from cellular level. Therefore, test animal *Heteropneustes fossilis* (Singhi) were treated with Zinc (II) Sulphate Heptahydrate ($ZnSO_4.7H_2O$) and *Psedumonas.* These stressors very commonly found in the water bodies of Bangladesh. Treated *Heteropneustes fossilis* (Singhi) was used to obtain tissue for conducting SDS-PAGE. SDS-PAGE was applied to protein samples to linearize proteins. A dominant band of 70 kDa was found in the gill after chemical exposure and after biological exposure another 70 kDa was expressed in kidney. Presence of 70 kDa suggests that HSP70 can be the ideal stress marker. The present study also indicates that the use of stress protein for biomarker is easy to use and sensitive for assessing water quality.

Keywords: *HSP70, stress protein, biomonitoring, environmental stressor*

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1. Introduction

Now a day, appraisal of water quality is based only on chemical measurements and analyses of the water itself though early detection of changes in the quality of water resources, especially those impacted by anthropogenic contaminants, is of primary concern to individuals. Those charged with managing water resources rely heavily on chemical, physical and bio monitoring techniques. A need exists for a rapid means of assessing the "health" of water bodies in Bangladesh. A reliable, field applicable method which determines stress levels in fish could provide resource managers with a valuable tool to determine if chemicals from point and non point source pollution are adversely impacting aquatic systems. This is possible with the use of biotests or biomarkers, e.g. investigations of the induction of heat shock proteins (proteotoxicity evaluate) [1]. Likewise, with proper validation it may be possible to determine if fish are approaching a level of stress nearing a threshold above which adverse impacts on growth and reproduction will occur.

Heat shock protein (HSP) genes are highly conserved as well as are constitutively expressed in all tissues in response to stress [2]. Stress disrupts the regular conformation of proteins demanding the aid of HSP to regularize the effect. In fish, stress could be due to any minor change in the environment. Fish are exposed to many kinds of stressors such as microbial infection, toxic exposure, traumatic damage, radiation, or nutritional deficiency. Therefore, the modulation of HSP genes is more apparent in fish and can be studied as molecular biomarkers of stress [3] because under adverse physiological conditions, there is a notable elevation of these proteins [4].

The most devastating stressor that can modulate the regular physiological activity of fish is microbial infection [4]. Activity of HSP is not only limited to the post-infection stress response but also can modulate the immune system as well [5].

2. Materials and Methods

2.1. Fish

Heteropneustes fossilis (Singhi) were studied in the laboratory tanks of constant temperature, 28℃. The oxygen content in the water was maintained throughout the experiment by an aerator, so that oxygen was not acting as a limiting factor. The fishes were fed with pelletized meal containing groundnut oil cake, Bengal gram powder and rice bran. The fishes were acclimatized to laboratory conditions such as feeding and temperature for a period of 15 days prior to the initiation of the experiment. Fish of both sexes were used without discrimination. Table 1 shows length of the fish varied from 12.2 to 13.9cm and the weight 8.6 -13.44 g.

Table 1. The length and weight of Singi fish *(Heteropneustes fossilis*)

Parameters	Range	Mean
Length (cm)	12.2 -13.9	13.05
Weight (gm)	8.6 -13.44	11.02

2.2. Heat Shock Exposure

After acclimatization, fish were moved to a similar tank where the water previously heated up to 34°C, and were left for two hours. Then, the fish were moved back to the tank of normal temperature and were left for two more hours. Blood samples were collected to monitor glucose level using glucometer and fish were anaesthetized by putting on the dry ice then dissected to obtain liver, heart, muscle, spleen and gill. Tissues were kept directly with dry ice then keeping under -70℃ for the next analysis.

2.3. Zinc (II) Sulphate Heptahydrate ($ZnSO_4.7H_2O$) Exposure

Forty fish were exposed to different concentrations of Zinc sulfate to determine LC_{50} value ten fish each. There is a tank for control has ten fish. The experiment lasted upto 72 hrs. The LC_{50} value is shown in the Table 2.

Cumulative mortality rate is shown in Figure 1.

Thus the survived fish were anaesthetized by putting on the dry ice then dissected to obtain tissue. Tissues were kept directly with dry ice then keeping under -70℃ for the following assays.

2.3. Exposure to biological stressor

To initiate bacterial infection caused by *Psedumonas* in Singhi fish, the required number of cfu/ml in water is more than 1.5×10^3 to 8.6×10^3. Before applying pathogens, it was essential to quantify the number of living pathogens that could infect Singhi. For this purpose, some of the pathogenic bacteria were incubated in nutrient l*ysogeny broth* to take the comparative optical density. This optical density helped to measure the amount of pathogen should be applied. Table 3 gives information amount of bacterial cells at specific optical density.

Table 2. LC_{50} values of $ZnSO_4.7H_2O$ at 24h, 48h and 72h for Singi fish *(Heteropneustes fossilis)*

Time of Exposure(Hours)	LC_{50} Values (ppm) for $ZnSO_4.7H_2O$	LC_{50} Values (ppm) for Zn
24	335	76.21
48	316	71.89
72	297	67.57

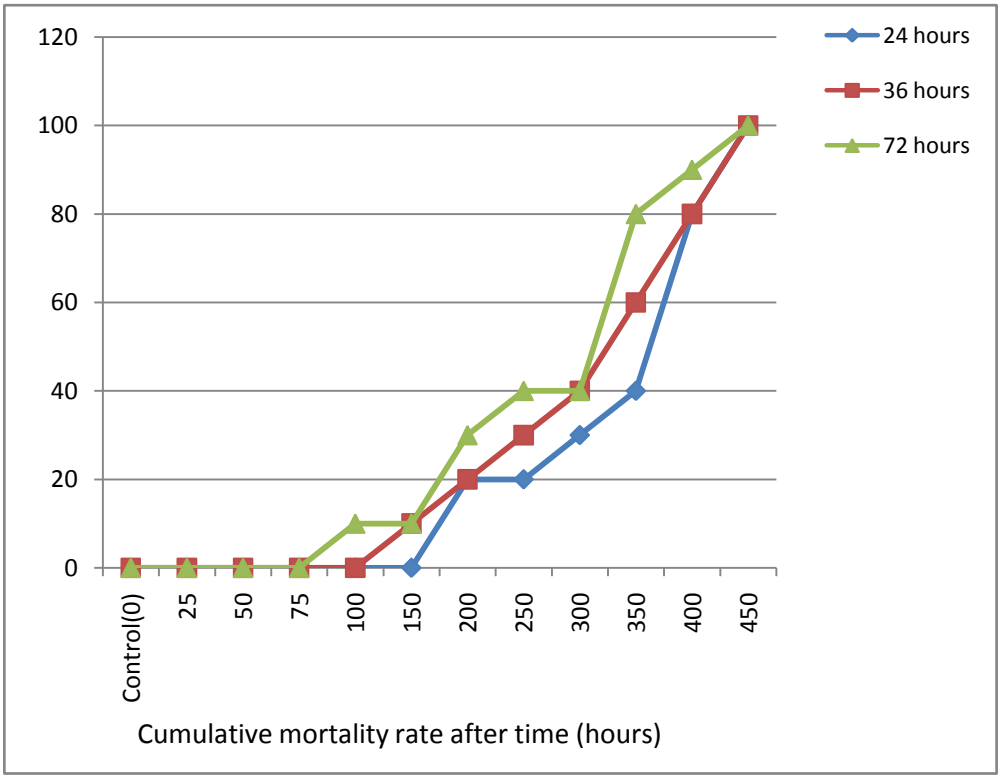

Figure 1. Cumulative mortality rate after time (hours)

Table 3. Required number of *Psedumonas* to initiate bacterial infection

No. of samples	OD after1hr 30min	No Of cfu /ml	OD after 5hr 30min	No Of cfu /ml	OD after 8hr 30min	No Of cfu /ml	OD after 24hr/2	No Of cfu/ml
1	0.03	2.6×10^6	0.396	2.4×10^8	0.885	7.8×10^8	0.696	6.7×10^{12}
2	0.127	2.5×10^6	0.419	2.16×10^8	0.628	5.8×10^8	0.581	1.70×10^{12}
3	.001	4.5×10^5	0.023	2.88×10^7	0.156	3.6×10^8	0.706	5.0×10^{12}
4	.011	2.6×10^6	0.429	2.016×10^8	0.605	4.4×10^8	0.617	6.0×10^{12}
8	.002	2×10^6	0.410	3.04×10^8	0.556	7.4×10^8	0.643	7.6×10^{12}

So, from the table, it is obvious that 10 ml of nutrient broth was applied after 4hrs incubation at 37℃. It raised specific optical density of different samples to 0.2 to 0.25 which were actually containing approximately 10^6 - 10^8 cfu/ml. So it can be said that the applied amount of pathogenic bacteria in aquarium was far higher than the required number of pathogens to initiate infection. Thus fish were stressed biologically and tissues were kept directly with dry ice then keeping under -70℃ for the following assays.

2.4. Homogenization

Tissues from fish were homogenized in 1ml tris-saline (1% SDS) by glass polytone. The homogenate is heated in water bath at 90℃ for 5 minutes, and then centrifuged at 14,000 rpm for 10 minutes. The supernatant was obtained and kept in refrigeration until use.

2.5. SDS PAGE

Gel concentration (%T) was selected according to the expected protein size (>100 kDa) so that the proteins of interest are resolved. Therefore A 10% separating gel was used. A 10% separating gel was constructed by mixing the distilled water, lower gel buffer along with 10% SDS and acrylamide solution. 10 ml of stacking gel mix was sufficient, however for the sake of accuracy it was preferable to make 20 or 30 ml. Excess was rinsed and tossed into a wastebasket after it polymerized. The sample was then carefully loaded onto the wells using a micropipette, one sample to one well. Hamilton syringes worked well for loading samples into the wells and the amount was limited to 10 µl. The tip of the micropipette was held at an angle just inside the well to minimize sample mixing with the reservoir buffer. The gel was run at 100 volts. In this condition, the electrophoresis apparatus was kept undisturbed for at least 1 hour and 45 minutes for the smaller unit, until the tracking dye (bromophenol blue) reaches to the gel bottom.

A commonly used stain for detecting proteins in polyacrylamide gels is 0.1% Coomassie Blue dye in 50% methanol, 10% glacial acetic acid. Excess dye was washed out by 'de-staining' with acetic acid, also with agitation. For more efficiency, de-staining been done in two steps: starting with 50% methanol, 10% acetic acid for 1-2 hours, later using 7% methanol, 10% acetic methanol to finish. The first solution shrinks the gel, squeezing out much of the liquid components and the gel swells and clears in the second solution. Properly stained/de-stained gels should display a pattern of blue protein bands against a clear background. The gels was dried down and photographed for analysis and documentation. The original dye front, consisting of bromophenol blue dye, disappeared during the process. In fact, bromphenol blue acted as pH indicator which turns light yellow under acid conditions, prior to being washed out.

3. Result

Blood glucose level was found to be higher for treated fish ranging from 2.7 at 24 hour to 9.7 at 72 hours with increasing concentration of Zinc (Table 4).

After finding the glucose level elevated and the fish were stressed, they were sacrificed and the stress protein was identified. A dominant band of 70kDa was found in the gill of Singhi after each chemical exposure. But was absent in any other organ every time when treated chemically. (Figure 2)

Table 4. Changes in Glucose level by $ZnSO_4$

Conc. of $ZnSO_4$ (ppm)	Blood glucose level (mmol/L)		
	24h	48h	96h
Control	4.35	4.67	4.99
25.00	5.21	5.32	5.57
50.00	5.10	5.60	6.99
75.00	6.32	6.76	7.19
100.00	6.49	7.78	8.80
150.00	7.98	8.30	8.75
200.00	8.30	8.45	8.98
250.00	8.77	9.28	9.86
300.00	9.10	9.35	9.99
350.00	10.32	10.58	11.32

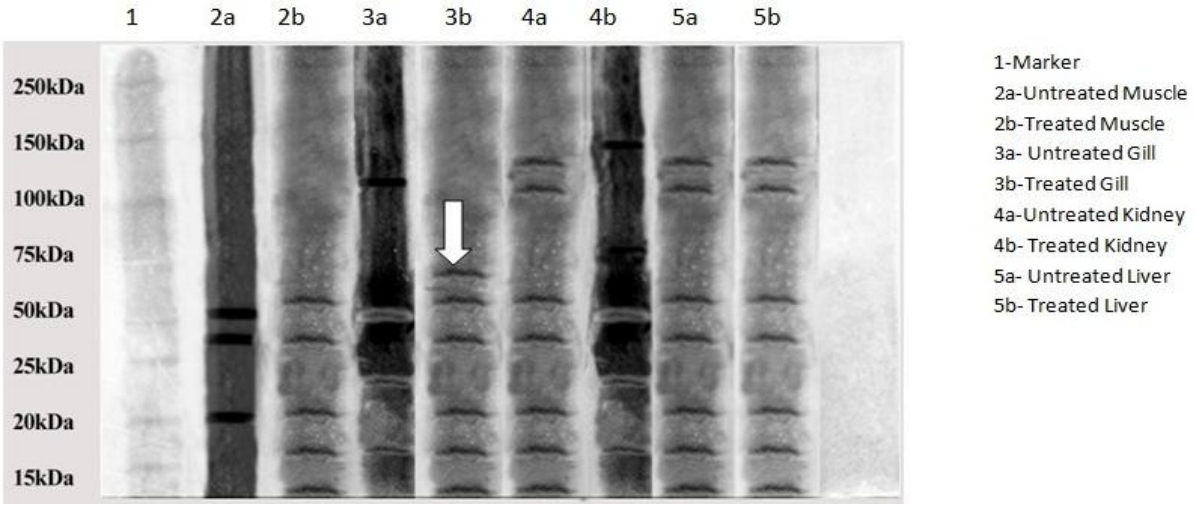

Figure 2. Banding pattern of *Heteropneustes fossilis* after Chemical exposure

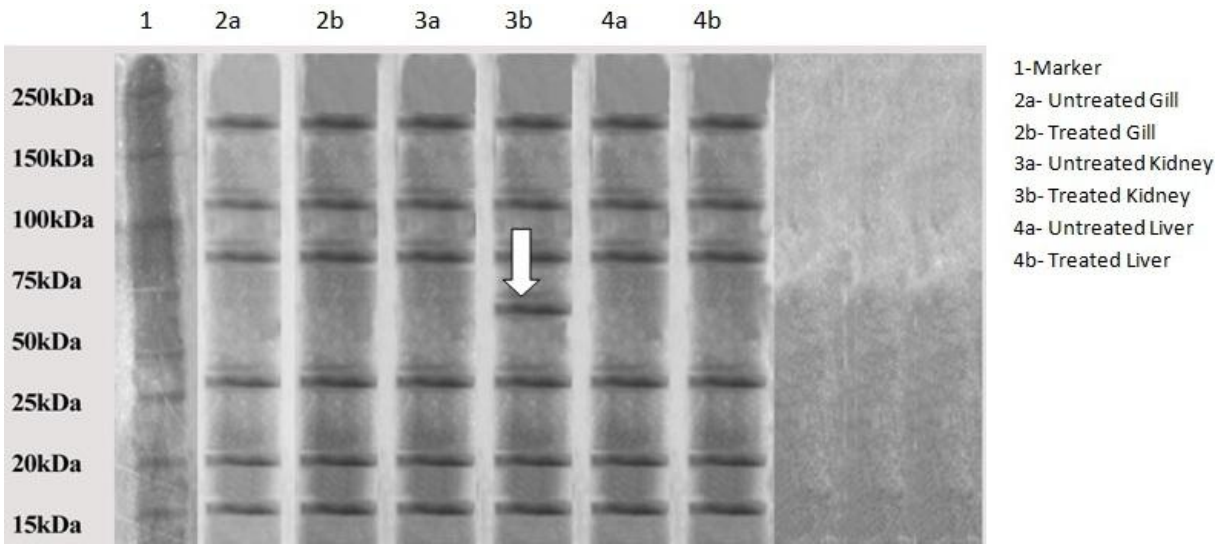

Figure 3. Banding pattern of Heteropneustes fossilis after biological exposure

Surprisingly, when stressed biologically with *Psedumonas*, another 70 kDa was expressed in kidney (Figure 3). Any of these response was absent in the control.

4. Discussion

Stress proteins mitigate the impact of stress in individuals and these mechanisms are becoming well understood at the level of model proteins, but less is known at the level of the cell, tissue, organ and whole organism [2] Therefore studies of stress protein response in organs with high metabolic activity, like kidney and gills, are of higher interest. This provides the first evidence that Hsp70 in both gill and kidney. And that certainly acts as a biochemical marker linked to proteotoxicity in Singhi fish. After the chemical treatment, the gills exhibited presence of the Hsp70 levels in treated fish. HSP70 was also found in gills of juvenile rainbow trout exposed to metals in the water or feed [6]. However, they are the primary organ to be affected and, therefore, the proteotoxic effect was likely to occur in more prominent way as in the kidney in case of biological treatment. Similarly, with respect to biotic stress tolerance, Hsp70 has also been shown to protect the brine shrimp [7,8,9] induced expression of Hsp70 gene was noticed in sea bream liver tissue at 36 h post-infection with Vibrio alginolyticus [10]. The induction was not limited to fish, but higher expression was also reported in infected crabs, clams, and shrimps as well during Gram-negative bacterial exposure [11,12,13].

Therefore, the present study points out only the initial but pertinent information on aspects of the response of fish to environmental stresses. This result encourages further research in this topic of environmental stress physiology since long term studies in the field suggested seasonal changes in heat shock response of fish [14]. The expression of HSP70 is regulated by a heat- shock promoter. Its transcription is activated by changes in body temperature as well as a variety of other stresses. For this reason, HSP70 is considered to be a useful marker protein of the stress conditions of animals [15].

Biomarkers refer typically to physiological or biochemical responses that serve as sensitive indicators of exposure to contaminants and or sub lethal stress. Whereas the response to environmental perturbations begins at cellular level, the use of stress proteins as indicators provide an early warning to prevent damage to higher organizational levels. The relationship between exposure to stressors such as heavy metals or environmental toxins and synthesis of HSPs has the potential to act as a significant indicator of environmental stress. A major drawback of most of the currently available indicators of stress in fish, such as plasma cortisol concentration or haematocrit, is that they are highly vulnerable to handling and sampling effects. The results of this research suggest the use of stress proteins as biomarkers may serve as an indicator of environmental perturbation in situations where the response to stress is chronic, sub lethal exposures and it is still possible to prevent the biological consequences of exposures which affect organismal or higher organizational levels. In this case, the persistence of the stress response may correlate with the intensity of the stressor. However, continuous exposure to severe environmental stress may lead to a physiological state from which the animal can no longer maintain the stress protein response and recover and ultimately death results. The effects would already be observed at the organismal level, but the stress protein response would have ceased. Therefore, data from the current experiment suggest that the ideal stress marker may be met by examining the accumulation of HSP70 in tissues. This indication is presented after exposure to environmentally biological and chemical contaminants. The present study also indicates that the use of stress protein as biomarker is very easy to use, practical, rapid, and sensitive for assessing water quality.

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