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Zebrafish Response to Extreme pH: Survivability and Expression of Core Nonsense-Mediated mRNA Decay Factors

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Abstract

Stress plays an essential role in maintaining fish health. The health and survival of fish strongly depend on stress, through which they display their ability to maintain stability under environmental stressors and physiological fitness. Fish react to environmental stressors by performing several biological changes when their water surroundings change the pH levels. The hypothalamus activation marks the starting point of major neurological and metabolic changes that affect overall physiologic equilibrium. Fish act as sensitive aquatic organisms that get stressed strongly by their environmental conditions, since even small changes can result in severe stress reactions. This study uses Zebrafish (*Danio rerio*) as test subjects to measure pH-stress effects. The fish were placed under different pH conditions of 4.2 (acidic) while monitoring both short-term and prolonged health effects caused by exposures to 8.2 (alkaline) and 10.2 (highly alkaline). The gill structures, along with the respiratory tract, presented visible harm because these tissues play essential roles in gas exchange and osmoregulation. The main purpose of this research is to examine how nonsense-mediated messenger RNA decay Neuromuscular Disease (NMD) acts as a vital eukaryotic surveillance system in zebrafish exposed to pH stress. The surveillance mechanism known as NMD completely degrades defective mRNA transcripts to guarantee correct gene expression functions. The research concentrated on core NMD transcript turnover rates under abnormal pH conditions to understand molecular stress responses at the single-celled level. This research reveals that exposure to sub-lethal pH conditions leads to the deterioration of physiological responses along with molecular functions that impair nonsense-mediated messenger RNA decay operations. Homeostasis failure occurs because of these disturbances, thus increasing environmental vulnerability and resulting in elevated mortality rates for fish populations. This research demonstrates the vital role of water quality, particularly pH, because it controls fundamental molecular mechanisms that determine fish health outcomes and survivorship.

Major Findings: Exposure to extreme pH levels was found to be detrimental for the survivability of Zebrafish, and they are sensitive to both acidic and alkaline stress. These conditions were in response to which their expression of key nonsense-mediated mRNA decay (NMD) factors. UPF1, UPF2, UPF3 were significantly altered, suggesting a molecular adaptation mechanism. Since these results show that the NMD pathway might have an essential role in zebrafish stress response and resilience to environmental pH extremes, modulating NMD activity will likely affect the response to environmental pH extremes in these fish.

Keywords: Fish Health, Nonsense-Mediated Messenger RNA Decay, pH, Toxicity, Zebrafish

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1. Introduction

Water parameters are essential for the survival of aquatic organisms, mainly different fish. Any change in the water parameters creates a stressful environment for them. Fish stress is considered an abnormal condition for physical or mental awkwardness[1]. The stress response triggers the release of stress-related hormones and results in behavioural and physiological changes[2]. The responses are categorised into primary, secondary, and tertiary reactions. Stress can be short-term or chronic, and severe stress can lead to illness and even fish death[3]. pH fluctuations represent a stressor for aquatic life like fish. Sudden pH changes can kill aquatic organisms, disrupting acid-base balance and ion regulation. Fish are highly stressed by pH fluctuations, which upset their internal physiological balance and general health. Fish species have developed to live in particular pH ranges suited to their natural environments[4]. The fish are unable to maintain the body's homeostasis at sudden or drastic variations from these ideal pH levels[5]. Long-term changes in pH can upset the delicate equilibrium of the aquatic environment and cause various physiological and behavioural changes in fish. Fish can have extreme stress reactions when exposed to drastic pH changes. As a result, there are instant physiological changes due to disturbances in ammonia excretion, ion control, and acid-base balance. They may have difficulty adjusting to the new environment, may display unpredictable behaviour, faster breathing rates, and changed swimming patterns[6]. Changes in pH level influence the solubility of dissolved gases, such as oxygen, which affects fish respiration. Fish may experience respiratory stress and even suffocation due to different pH values that limit oxygen availability. Fluctuation in pH affects the ion control processes essential for maintaining osmoregulation and causes physiological disturbances[7]. Fish that are stressed by pH often react behaviorally, showing symptoms of distress, including lethargy or irregular swimming patterns[8]. Keeping pH levels consistent and appropriate in aquatic ecosystems is crucial for reducing stress and advancing fish populations' health and well-being. Stress can result from abrupt changes in their physiological equilibrium or from extraordinarily high or low levels of acidity (low pH) or alkalinity (high pH). Different pH levels also affect the fish at the genetic level. Stress modulates the post-translational modification, mainly the mRNA surveillance mechanism[9,10].

The NMD is a highly conserved cellular surveillance mechanism that recognises a Premature Stop Codon (PTC) within an mRNA transcript and degrades it, thereby preventing the production of a truncated, possibly harmful protein[11,12]. In the context of normal gene expression, NMD also has a role in quality control in regulating the processing of specific endogenous transcripts. NMD controls ≃ 10% of wild-type transcripts, impacting biological processes like cell cycle progression, signal transduction, and development[13,14]. Ribosomes that prematurely stop translation cause NMD. This action results in the formation of complex surveillance factors, which mainly destroy an mRNA containing PTC[19]. The surveillance complex works to speed up the destruction of mRNAs with PTCs by involving standard cellular mRNA decay machinery[15,16]. As a vertebrate model of NMD with genetic manipulability, transparent embryos and rapid development, Zebrafish (*Danio rerio*) is a powerful tool[17,18]. Key NMD pathway components that are functionally conserved in zebrafish (e.g., UPF1, UPF2, UPF3) can be dissected in embryogenesis, in neural development and the regulation of transcriptome. Additionally, Zebrafish models aid in figuring out the molecular bases of human genetic disorders caused by NMD dysfunction, and can be used to examine possible treatments for NMD and more broadly, as potential therapeutics[19,20]. The *Danio rerio* is a miniature freshwater teleost member of the minnow family. This tiny creature is widely used as an animal research model for toxicological studies[21]. There is a presence of striped lines on the whole body like a Zebra, so they are also known as Zebrafish/ Danio Zebra. Zebrafish have 70% of their genetic level to other higher eukaryotic animal models like humans, and thus it is a good research model[22]. This research aims to discover how Zebrafish respond to extreme pH conditions from both the survival perspective and the molecular regulation of the NMD pathway. To explore the potential role of mRNA surveillance in environmental stress responses, Zebrafish expressing key NMD factors were exposed to acid and alkalinity, by which we can start to unravel. In addition to providing important indications of how vertebrates generally adapt to challenging ecosystems, this work adds important additional knowledge about Zebrafish physiology, contributing to the field of environmental molecular biology.

2. Methodology

2.1 Zebrafish Facility

Hundreds of adult wild-type Zebrafish (5-6 months post-fertilisation) were procured from the Central Institute of Freshwater Aquaculture (ICAR-CIFA). The wildtype adult Zebrafish weighed 0.9±0.1g. They were acclimated

to laboratory settings for eight weeks in a 30L stock tank equipped with good-quality filters. The aquarium water conditions were maintained daily with a pH of 7±0.2, temperature of 27±1°C, and conductivity of 490-510 S/cm. The aquarium was kept on a 14-hour light/10-hour dark cycle, with lights turned on at 9:00 AM [1]. They were fed commercial processed dry feeds (Optimum Tropical Fish Food - Mini Pellet) twice daily during the trial. The setup was cleaned periodically to prevent infections. After the acclimation period, the actual experiment was initiated.

2.2 Applying Abiotic Stress Conditions

For the experiment, the seven 8-inch bowls with a 3L water capacity were placed for the experimental setup. Three wild-type adult Zebrafish weighing 0.9±0.1g were placed in each bowl under the same circumstances as in the stock aquarium, along with an aerator pump. The first part of the experiment measured the zebrafish's tolerance to different pH water levels for 72 hours. There was a glass bowl with tap water as the control. In three glass bowls, acidic water concentrations of pH 4.2, 5.2, and 6.2 were built up with tap water using acetic acid. Similarly, sodium hydroxide was used to prepare alkaline water using tap water in another three glass bowls to create a range of concentrations, including pH 8.2, 9.2, and 10.2. The pH range of the tap water was 7.2–7.4, with 7.2 acting as the reference point. Three wild-type zebrafish were chosen at random and quickly moved into each of the experimental bowls that had aeration. CHEMI LINE Technologies, a portable pH meter, was used to measure the pH of the water. The pH of the water was measured twice a day in the morning and evening, and the pH of the basic and acidic baths was tested and kept within a ±0.1-unit range. Individual fish survival periods in the different pH concentrations were noted. The fish was considered dead based on its complete lack of response to external stimuli and its opercular beats.

2.3 Feeding

Fish were fed with a commercial floating feed. Feeding was done twice a day, with 2 counts of feed/fish.

2.4 Histology of Zebrafish

The dead Zebrafish were dissected immediately after death. Then, the different parts of the Zebrafish were seen under the microscope, and pictures were taken. Then, all the images were compared with the images of the control fish to distinguish the difference between each stress concentration level.

2.5 RNA Isolation and sqRT-PCR for UPFs

The tissue of stress-affected dead Zebrafish was collected, and 100mg of tissue was used for RNA isolation. The control zebrafish at pH 7.2 also went through the process of RNA isolation. According to the manufacturer's instructions (Qiagen RNA Isolation Kit), total RNA was extracted, and 2 units of DNase were used to remove the genomic DNA. First-strand cDNA was synthesised using 5µg of total RNA by RTase. RT-PCR was performed to check the expression of UPFs in the fish with and without stress conditions (Control).

3. Results

3.1 Behavioural Study

There was no mortality of naïve fish during the acclimatisation period before stress exposure. It was observed that the behaviour and morphology during the experimental days by comparing the stressed Zebrafish with the control. We noted the information and recorded the survival period of fish post-stress (Table 1). According to the observations, the concentrations of acetic acid and sodium hydroxide that resulted in pH values of 4.2 and 10.2, respectively, were the top fatal limits of pH for Zebrafish. Fish deaths were noted after exposure to different acidic and alkaline pH values (Table 1). Fish gills and skin were heavily coated in mucus, and the gills' respiratory epithelium was damaged mainly at acidic pH extremes. The fish showed hypoactivity while taking a diagonal position with its head toward the water's surface in the acutely acidic atmosphere (Table 2). It then became lethargic and occasionally shaken violently before passing away. When fish came across a deadly basic pH, they showed restlessness, swimming quickly and slapping their tail area.

Additionally, it was observed that fish exposed to pH 4.2 and 10.2 initially had slow swimming patterns and a pale appearance after about an hour. There was decolouration of the stripped line on the skin of the Zebrafish due to the extreme stress levels. Decrease the spontaneous activity of the fish towards taking food. Fish showing higher opercular ventilator movements, with possible open mouth (Hyperventilation). Between 24

and 48 hours of exposure, all specimens died at pH 4.2 and pH 10.2. On the other hand, 70% of the fish perished at pH 5.2 and 9.2 within 48 hours of exposure. The Zebrafish housed at all other pH levels, 6 - 8, exhibited less appreciable behavioural changes than other extreme pH conditions. During the observation period, some fish faced mortality. The post-stress behaviour information is given in the Table 2.

3.2 Comparative Studies of Zebrafish Exposed to Different pH

All the Zebrafish exposed to different pH levels showed different morphological changes compared to the control. There were clear blue fluorescent lines on the body in control, while in highly acidic exposure, these lines were not seen in the body of the Zebrafish (Figure 1). In the alkaline water exposure, stripped lines were present on the bodies of Zebrafish (Figure 1). The black eye lens is discoloured into fully white at a high acidic pH of 4.2, but less at pH 5.2 and 6.2 (Figure 1). The discolouration of the eye lens is not seen in fish at pH 8.2, 9.2, and 10.2. In the red circle, there are also black spots on the gill area of the fish's body at acidic pH, which shows that the gills may be highly affected by the acidic pH.

After the exposure to a stressful environment, brown colour was seen on the body of the Zebrafish, which implies the tissues present in those areas were affected by the stress-inducing factors (Figure 2).

During the observation days, the fish showed different activities before death. When the fish died, they were identified by checking their response to stimuli and the movement of different parts, mainly the gills. Once a fish is found dead, it is immediately dissected, and the different parts are generated into pictures for analysis (Figure 3). All the differences seen in the fish body are compared with the control zebrafish. There was a distinct difference seen in the case of eyes, gills, and swim bladder in acidic pH 4.2, 5.2, and 6.2. In the case of alkaline pH 8.2, 9.2, 10.2, the posterior intestine is highly affected as that tissue rots and is seen as a black, dead tissue (Figure 3).

Subsequently, the staining images of the gills were compared for different pH. After the dissection, the collected gills were stained by the Hematoxylin Eosin (H and E) staining process and seen under the microscope at a scale bar of 10x. In dissection, the zebrafish's gills were

Table 1. Mortality record of fish after exposure to pH stress

Experimental Sets	pH	Mortality after stress (in hours)			
		Zebrafish (N=3)	Fish 1	Fish 2	Fish 3
Control	7.2	3	---	---	---
Set I	4.2	3	15	20	24
Set II	5.2	3	28	40	48
Set III	6.2	3	36	60	---
Set IV	8.2	3	40	65	---
Set V	9.2	3	28	36	45
Set VI	10.2	3	14	18	23

Table 2. Effect of pH on the physiology of Zebrafish

Experiment Setup No.	Stress Agent (pH)	Symptoms observed in Zebrafish after stress
Control	7.2	Greyish skin colour with striped lines, Normal behaviour.
Set I	4.2	Lighten skin colour, hyperventilation, Decrease in spontaneous activity, and swimming slowly.
Set II	5.2	Lighten skin colour, hyperventilation, Decrease in spontaneous activity, abnormal swimming behaviour.
Set III	6.2	Slight change in skin colour, normal ventilation Decrease in feeding activity, abnormal swimming behaviour.
Set IV	8.2	Slight change in skin colour, normal ventilation Decrease in feeding activity, abnormal swimming behaviour.
Set V	9.2	Lighten skin colour, hyperventilation Decrease in spontaneous activity, abnormal swimming behaviour.
Set VI	10.2	Lighten skin colour, hyperventilation A decrease in spontaneous activity, swimming at the bottom.

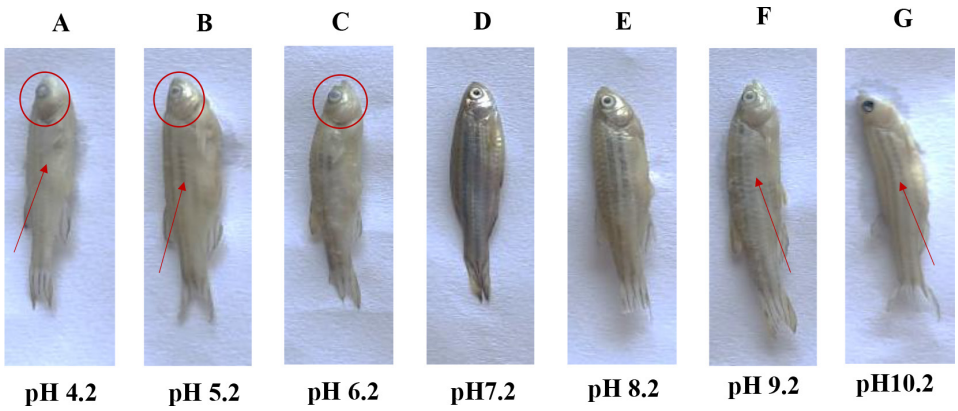


Figure 1. Dorsolateral side view of fish: The red arrow shows the discolouration of pigmented lines in the skin of zebrafish. The red circles show discolouration of the black eye lens.

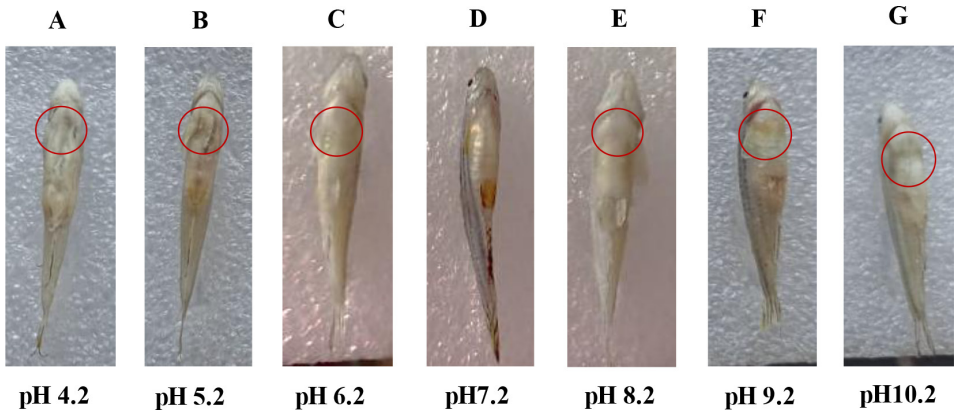


Figure 2. Ventral side view of fish: The red circle indicates the brown spots on the fish's body. An acidic pH shows that the gills and respiratory tract may be highly affected. The brown spots near the intestinal area are an alkaline pH.

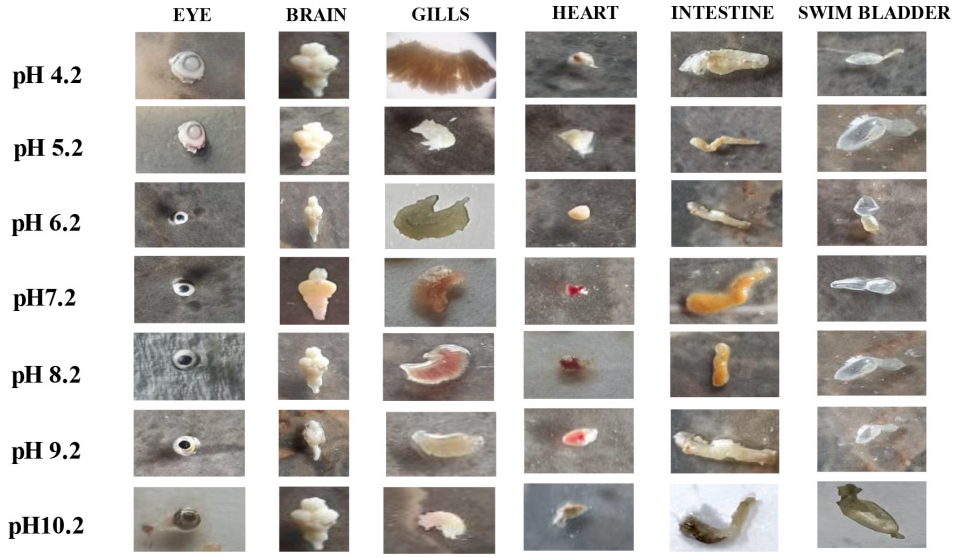


Figure 3. Comparative anatomy of Zebrafish exposed to stress: The dead Zebrafish (exposed to stress) were dissected and separated into different parts of the Zebrafish, as shown in an image. Different body parts like the eyes, brain, gills, heart, intestine, and swimbladder of Zebrafish are exposed to different pH stress.

highly acidic as the primary and secondary laminae were degraded (Figure 4). From the images, it was identified that in highly acidic conditions, the primary and secondary laminae were degraded and detached due to the effect of highly acidic conditions. The higher degeneration is seen in the case of pH 4.2 environments.

The fish reached the stage of mortality as a result of exposure. Due to the stressful conditions, the fish died at different pH levels. The mortality percentage is slightly higher in acidic conditions than in alkaline conditions (Figure 5). There was no mortality in the control. The detailed information on the mortality percentage in the different pH is described below (Figure 5).

3.3 Multiple Sequence Alignment of UPFs

Multiple sequence alignment generates links between closely related genes or proteins. It often leads to fundamental biological insight into sequence-structure-function relationships of nucleotide or protein sequence families. Multiple sequence alignment has a unique advantage because it reveals more biological information than many pair-wise alignments. It allows the identification of conserved sequence patterns and motifs in the whole sequence family, which cannot be detected by comparing only two sequences. According to multiple sequence alignments, NMD core factors such as UPF1 are highly conserved (Figure 6).

3.4 Differential Expression of the Core NMD Factors

All the dead fish were collected and processed for RNA isolation during observation days. Later, the cDNA was used as a PCR template to amplify a given set of genes, including UPF1, UPF2, UPF3, and Actin (internal control). Strikingly, it was observed that UPF1, UPF2, and UPF3 expressions were elevated after exposure to extreme pH. Actin was used as an internal control in the experiment (Figure 7). The band intensities were used to

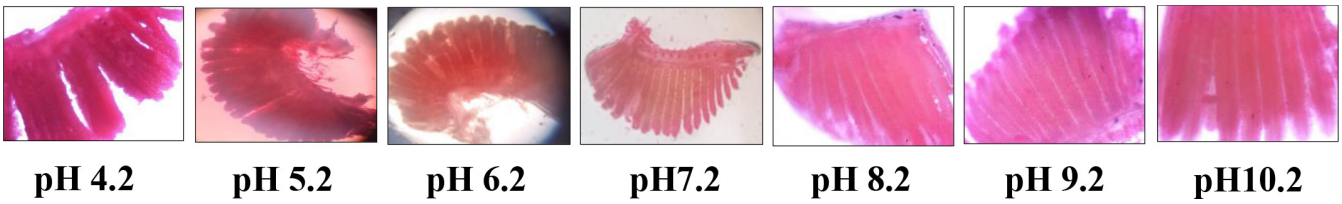


Figure 4. Histological study by H and E staining of Zebrafish gill: Representative images are a view of Zebrafish gill following hematoxylin and eosin staining. Magnification and scale bar 10x corresponds to different stress.

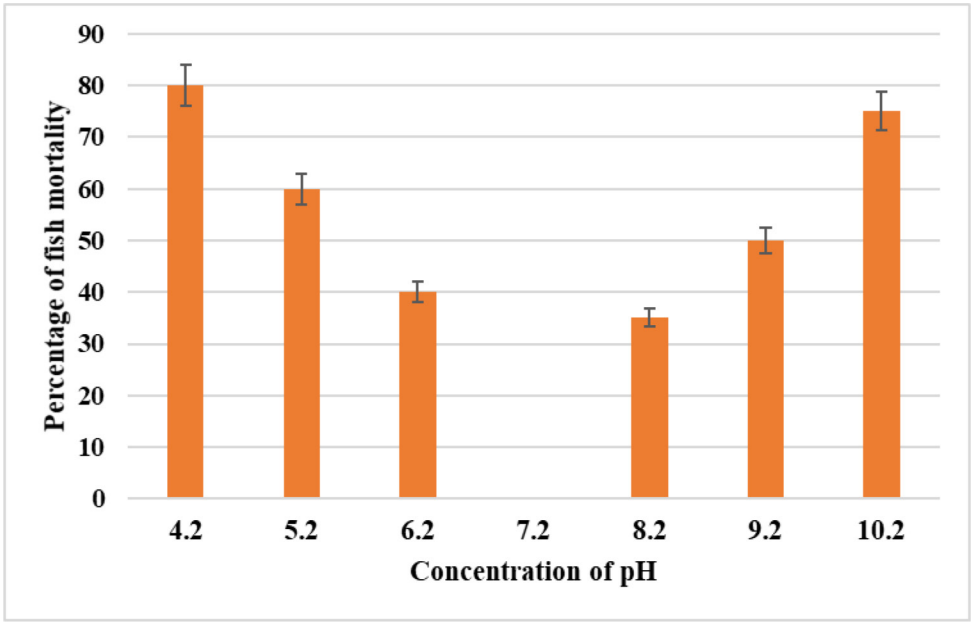


Figure 5. The relation between the concentration of pH and the percentage of fish mortality.



Figure 6. Multiple protein sequence alignment shows the relatability of the *Danio rerio* (Zebrafish) gene with the same gene of other research organisms and multiple sequence alignment of UPF1. Residues that are 100% conserved are in red boxes. Similarity >70% is red. The figure was generated using Multalign and ESPript.

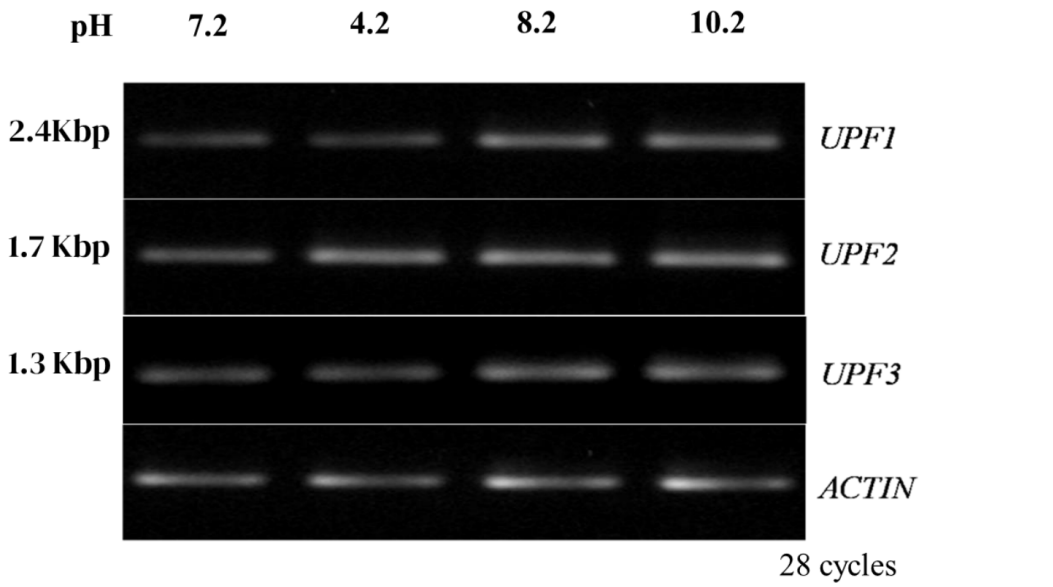


Figure 7. RT-PCR and agarose gel electrophoresis showing differential expression of UPFs in response to pH exposure.

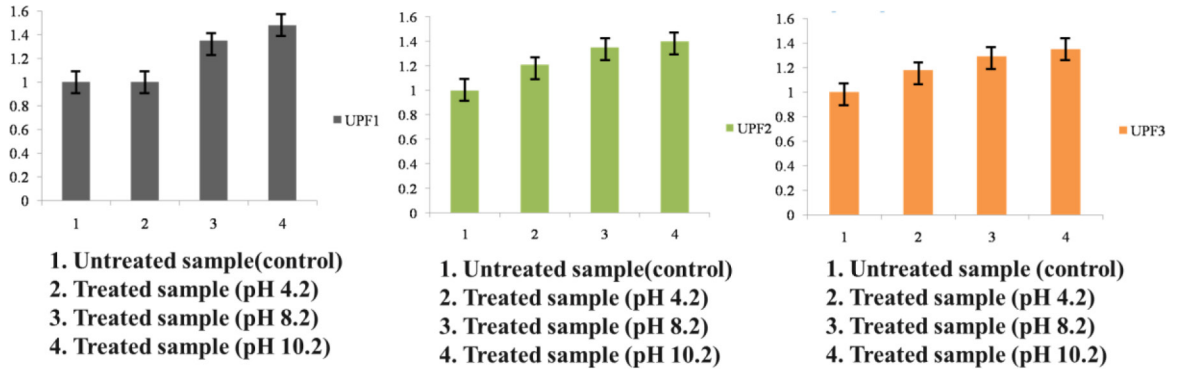


Figure 8. Fold-change in the level of UPFs upon exposure to different pH.

measure the density using Image J software, highlighting the nature of the relative expression of the given NMD transcripts under pH stress (Figure 8).

4. Discussion

These finds, that physiological stress and limited adaptability to such environments are observed in the direct correlation between increased pH concentration (both acidic and alkaline) and increased mortality rates and an inverse relationship between mortality time and pH concentration indicate that even slight change in pH concentration in nearshore habitats leads to increased physiological stress and mortality[23]. This stress response is a natural thing, however, when the environmental changes, like changes in pH are greater than can be handled by the fish's adaptive capacity, this becomes detrimental. Physiological disruptions in zebrafish exposed to alkaline (pH 8.2 and 10.2) and acidic (pH 4.2) conditions are observed and serve to highlight the sensitivity of zebrafish as aquatic organisms and of vulnerable key physiological structures, including gills and respiratory tissues. Damaged in pH stress, these tissues are critical to maintaining internal balance by way of gas exchange and osmoregulation, and the compromised health in general is indicated[24]. Moreover, this information can help in ecological risk assessments by providing molecular biomarkers for stress and survivability thresholds. The paragraph ends correctly by pointing to the need for further studies to identify the individual roles of NMD components such as UPF1, UPF2, UPF3 in the regulation of stress-responsive transcripts and the preservation from RNA damage. These findings support that environmental stress, especially pH fluctuation, plays an essential role in determining how fish health and survival, and molecular regulation are affected. The modulation of NMD core factors in these conditions is an important possible mechanism linking environmental stress and post-transcriptional regulation of genes[25]. Since excessive damage or misregulated transcripts may be countered by activating NMD, a cell strategy, its activation under pH stress may play a key role in maintaining mRNA integrity and proteome stability. Furthermore, the discussion in the paper suggests that even while the regulatory function of NMD in the context of environmental stress is still being defined, these findings can be used as an avenue for going further towards understanding RNA quality control in stressful contexts[26].

5. Conclusion

The outcomes of this experiment provide information that there was a direct relationship between mortality and concentration levels of pH. When concentration levels increased (both acidic and alkaline), the mortality rate was also amplified. However, there was a negative relationship between the mortality time and different concentration levels; when the concentration level increased, the mortality time decreased. The current investigation results show that the fish responded to both the primary effects of pH stress and the secondary effects brought on by stress. Also, behavioural changes are seen prominently, along with increasing stress levels. Most importantly, the expression of NMD core factors is altered when the Zebrafish is exposed to varied stress conditions, highlighting the effect of pH stress on the NMD surveillance mechanism. The regulation of NMD by environmental stress is also still in its early stages, and this is also the case for pH extremes. This is, however, plausible, as NMD has a vital role in mRNA stability and proteome quality, and thus, its involvement in stress response mechanisms is quite probable. Under such conditions, they might feel oxidative stress, changes in intracellular signalling, and modifications to RNA stability, all of which potentially affect NMD components expression/activation. This, therefore, represents an opportunity to examine the effects that environmental stress may have on NMD activity in Zebrafish, thereby providing new clues into how organisms regulate RNA quality control under stressful conditions. Additionally, information on the survivability of Zebrafish under extreme pH conditions is potentially useful for monitoring of the environment and ecological risk assessment, as it can give us data on physiological thresholds, tolerance limits and mortality patterns. While coupling studies of survival to gene expression of core NMD factors does produce a more complete understanding of the molecular adaptations to survival in the case of stress, work remains to be done. For example, if Upf1, Upf2, and Upf3 are upregulated under low or high pH conditions, this could suggest that NMD functions in a protective manner against stress-induced RNA damage or in the regulation of stress-responsive transcripts. Further investigation will shed the detailed molecular mechanisms in understanding the interlink between abiotic stressors and NMD regulation in eukaryotic model organisms and possibly will address the United Nations' Sustainable Development Goals (SDG), like SDG 14.

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8. Credit Authorship Contribution Statement

All the authors have made substantial contributions to the preparation of the manuscript. GKP: conceptualised and conceived the idea. RD and GKP: conducted experiments, data curation and writing. All the authors have read and approved the final manuscript before submission.

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