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Extreme pH Affects the Expression of the Core Nonsense-mediated mRNA Factors in Zebrafish

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Abstract
Stress plays an essential role in maintaining the fish health. Different stress agents' response initiates the hypothalamus's activation and subsequent changes in the neuroendocrine system, metabolism, and physiology. Fish are heavily influenced by their environment in various aquatic habitats. In the current study, zebrafish were exposed to varying pH levels, and the resulting stress responses were observed. The acute toxicity and chronic effects of extreme pH 4.2, 8.2, and 10.2 are effectively seen on zebrafish's gill. The current research primarily aims to shed light on the role of nonsense-mediated messenger RNA decay (NMD), a eukaryotic surveillance mechanism, explicitly focusing on the turnover of the core NMD transcripts in zebrafish exposed to extreme pH conditions. This study enlightens how water quality parameters like pH affect specific physiological and molecular events in zebrafish. In summary, exposure to sub-lethal pH concentrations influences zebrafish's physiological and NMD processes, resulting in zebrafish mortality.
Keywords: Zebrafish, fish health, pH, toxicity, nonsense-mediated messenger RNA decay

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. Stress in fish is considered an abnormal condition for physical or mental awkwardness. The stress response triggered the release of stress-related hormones and resulted in behavioural and physiological changes (Chu et al., 2019). The responses are categorized into primary, secondary, and tertiary reactions. Stress can be short-term or chronic, and severe stress can lead to illness and even fish death (Skomal et al., 2012). 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 (Sampaio et al., 2016).

The fish are unable to maintain the body's homeostasis at sudden or drastic variations from these ideal pH levels (Wedemeyer et al., 1990). Long-term changes in pH can upset the delicate equilibrium of the aquatic environment and cause various physiological and behavioral 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. Fish having difficulty adjusting to the new environment may display unpredictable behavior, faster breathing rates,

and changed swimming patterns (Schulte et al., 2014). 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 (Mallya et al., 2007). Fluctuation in pH affects the ion control processes essential for maintaining osmoregulation and causes physiological disturbances (Killen et al., 2013). Fish that are stressed by pH often react behaviorally, showing symptoms of distress, including lethargy or irregular swimming patterns (Portz et al., 2006). 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 (Panigrahi et al., 2020a, 2020b, 2020c). Among all the pathways involved in mRNA surveillance, abiotic stress mainly disrupts the nonsense-mediated decay pathway. The nonsense-mediated mRNA decay (NMD) is an evolutionarily conserved process for regulating the quality of mRNA that assures the accuracy of protein formation by isolating and destroying mRNAs that include premature termination codons (PTCs)(Patro et al., 2023; Das et al., 2024). As a result, the NMD process protects eukaryotic cellular mechanisms from any adverse conditions due to the buildup of truncated proteins (Jung et al., 2020). PTCs mainly contain nonsense codons. NMD controls $\simeq$ 10% of wild-type transcripts, impacting biological processes like cell cycle progression, signal transduction, and development (Panigrahi et al., 2021a,2021b,2021c).

Ribosomes that prematurely stop translation cause NMD to occur. This action results in the formation of complex surveillance factors, which mainly destroy a mRNA containing PTC (Panigrahi et al., 2024). The surveillance complex works to speed up the destruction of mRNAs with PTCs by involving standard cellular mRNA decay machinery (Sahoo et al., 2023; Sahoo et al., 2024). Extreme stress influences the expression of the core NMD factors such as UPFs (Up-stream protein factors; Sahoo et al., 2021a, 2021b). The *Danio rerio* is a miniature freshwater teleost and a minnow family member (Singleman et al., 2014). This tiny creature is widely used as an animal research model for toxicological studies (Khan et al., 2019). There is the presence of stripped lines on the whole body like zebra, so they are also known as zebrafish/danio zebra (Delcourt et al., 2018). Zebrafish have 70% genetic similarity to other higher eukaryotic animal models like humans; for this reason, zebrafish is a good research model (Khan et al., 2018). This study will use the zebrafish model to investigate how pH stress affects the mRNA surveillance mechanism and all tissues, such as the gill, skin, eye, and intestine. Hence, the present paper deals with the stress responses of zebrafish to changes in pH water.

Methodology

Zebrafish facility

Hundreds of adult wildtype zebrafish (5-6 months post-fertilization (mpf)) were procured from the Central Institute of Freshwater Aquaculture (ICAR-CIFA). The wildtype adult zebrafish were having weight 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 (Avdesh et al., 2012). They were fed commercial processed dry feeds (Optimum Tropical Fish Food - Mini Pellet) twice daily during the trial. We periodically clean the setup to prevent infection as once a weak. After the acclimation period, the actual experiment began.

Applying abiotic stress condition

For the experiment, the seven 8-inch bowls with 3L water capacity were placed for the experimental setup. Three wild-type adult zebrafish weighing 0.9 ± 0.1g were placed in each bowl with 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 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 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 function of opercular beats.

Feeding

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

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.

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 synthesized using 5 µg of total RNA by RTase. RT-PCR was performed to check the expression of UPFs in the fish with and without stress conditions (Control).

Results and Discussion

Behavioural study

There was no mortality of naïve fish during the acclimatization period before stress exposure. We observed 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 encountered a deadly basic pH, they showed restlessness, swimming quickly and slapping their tail area.

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

Experimental sets	pH	Zebrafish (N=3)	Mortality after stress (in hours)		
			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

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 decoloration of stripped line on the skin of zebrafish due to the extreme stress levels. Decrease the spontaneous activity of the fish towards taking feed. 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 behavioral changes than other extreme pH conditions.

During the observation period, some fish face mortality. We have given behavior post-stress information in the table below **(Table 2)**.

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 color with striped lines, Normal behavior
Set I	4.2	Lighten skin color, hyperventilation, decrease in spontaneous activity, and swim slowly.
Set II	5.2	Lighten skin color, hyperventilation, Decrease in spontaneous activity, abnormal swimming behavior.
Set III	6.2	Slightly change in skin color, normal ventilation Decrease in feeding activity and abnormal swimming behavior.
Set IV	8.2	Slightly change in skin color, normal ventilation Decrease in feeding activity, abnormal swimming behavior
Set V	9.2	Lighten skin color, hyperventilation Decrease in spontaneous activity, abnormal swimming behavior
Set VI	10.2	Lighten skin color, hyperventilation Decrease in spontaneous activity, swimming at the bottom.

Comparative studies of zebrafish exposed to different pH

All the zebrafish exposed to different pH 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 discolored into fully white at a high acidic pH of 4.2 but less at pH 5.2 and 6.2 **(Figure 1)**. The discoloration of the eye lens is not seen in fish at pH 8.2, 9.2, and In the red circle, there are also black spots on the gill area of the fish body at acidic pH, which shows that the gills may be highly affected by the acidic pH.

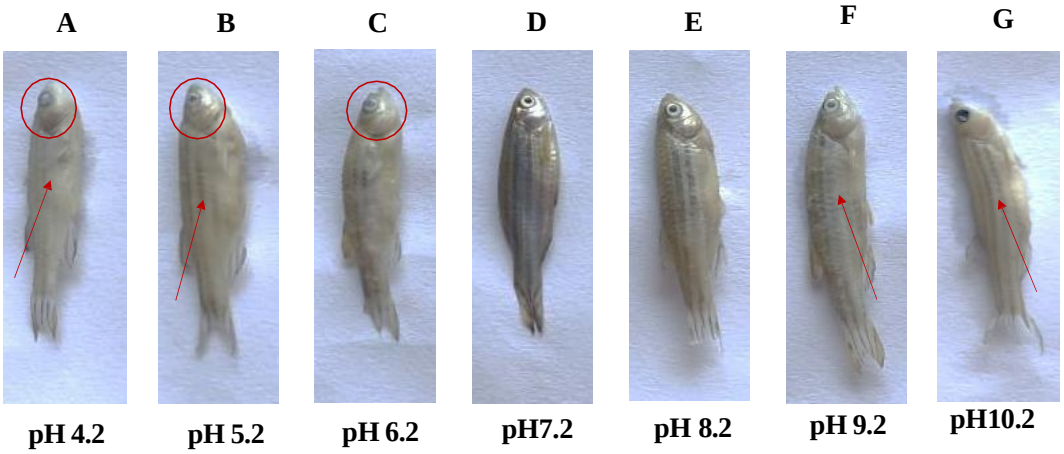

Figure 1. ***Dorso-ventral side view of fish The red arrow shows the discoloration of pigmented lines in the skin of zebrafish. The red circles show discoloration of the black eye lens.***

After the exposure to the stress environment, a brown colour was seen on the bodies of the zebrafish, which implies that the tissues present in that area were affected by stress-inducing factors **(Figure 2)**.

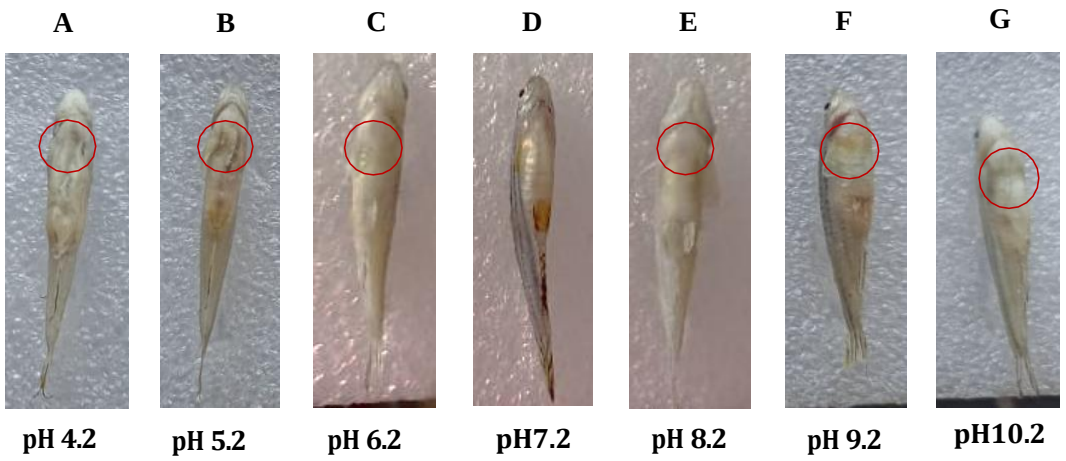

Figure 2. ***Ventral side view of fish: The red circle indicates the brown spots on the fish's body. The acidic pH shows that the gills and respiratory tract may be highly affected. The brown spots near the intestine area have an alkaline pH.***

During the observation days, fish showed different activities before death. When the fish was dead, it was identified by checking their response to stimuli and the movement of different parts, mainly gills. Once a fish was found dead, the immediately dissected fish was separated into different parts and generated pictures for analysis (**Figure 3)**. All the differences seen in the fish body are compared with the control zebrafish. A distinct difference was 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, and 10.2, the posterior intestine is highly affected as that tissue got rotten and is seen as black dead tissue (**Figure 3)**.

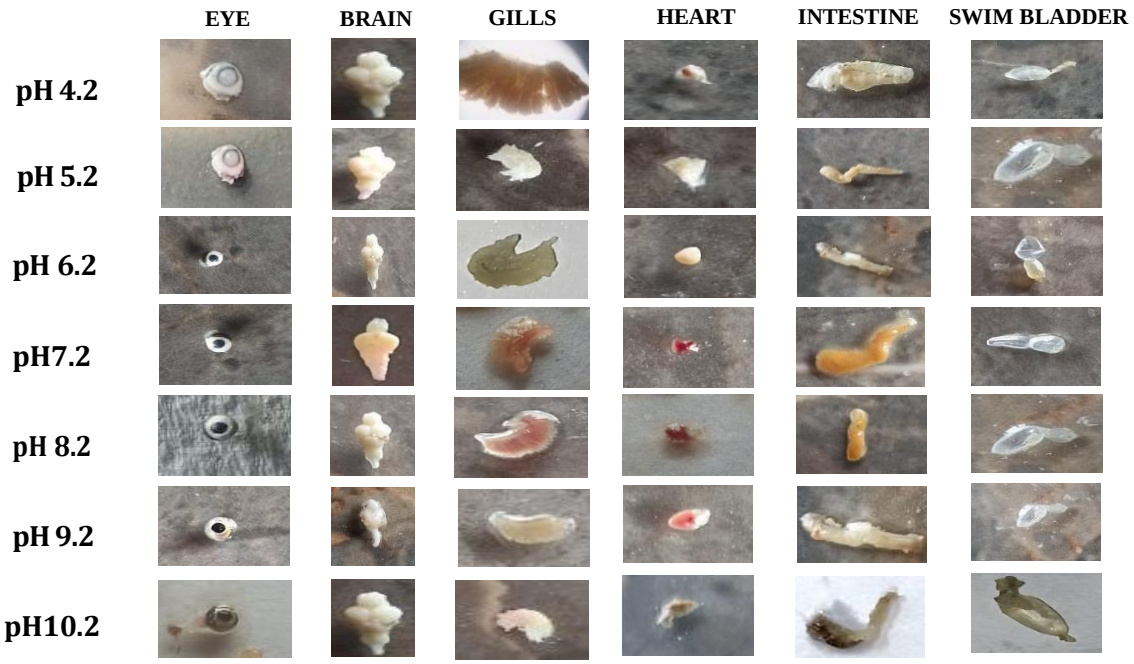

Figure 3. ***Comparative anatomy of zebrafish exposed to stress: The dead zebrafish (exposed to stress) were dissected and separated into different parts of the zebrafish, as shown in an image. Different body parts like the eyes, brain, gills, heart, intestine, and swimbladder of zebrafish are exposed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

Subsequently, the staining images of the gills were compared for different pH. After the dissection, the collected gills were stained by the H&E staining process and seen under the microscope in the scale bar of 10x. In dissection, zebrafish's gills were 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 attached to each other due to the effect of highly acidic conditions. The higher degeneration is seen in the case of pH 4.2 environments.

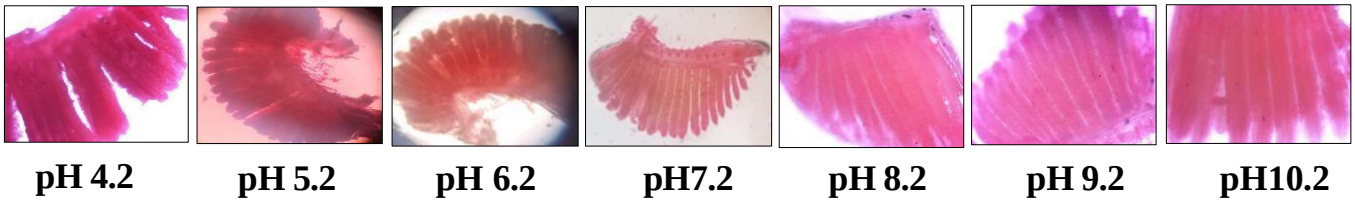

Figure 4. *A histological study by H&E staining of zebrafish gill: Representative images are a view of zebrafish gill following hematoxylin and eosin staining. Magnification and scale bar 10x correspond to different stress.*

The fish ended its density at the stage of mortality as the effect of exposure. Due to the stressor conditions, the fish died at different percentages and different pH levels. The mortality percentage is slightly higher in acidic conditions than in alkaline conditions (**Figure 5)**. There was no mortality in the case of control. Detailed information on mortality percentages in the different pH is described below (**Figure 5)**.

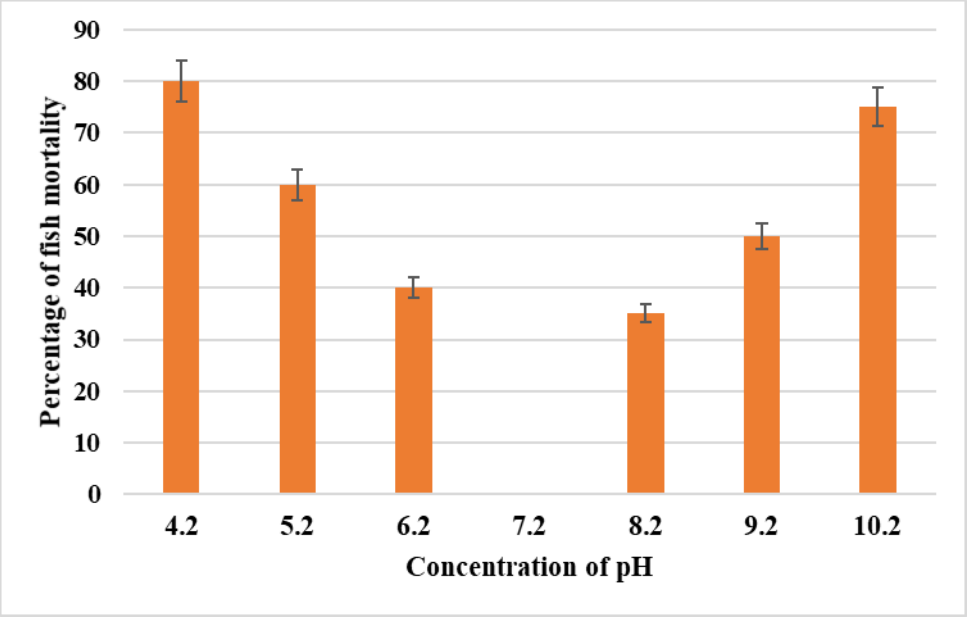

Figure 5. ***Showing the relation between concentration of pH and percentages of fish mortality***

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 UPF1is highly conserved **(Figure 6)**.

Figure 6. ***Multiple sequence alignment shows the relatability of the Danio rerio (Zebrafish) gene with the same gene of other research organisms. A) 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.***

Differential expression of the core NMD factors

All the dead fish are 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 measure the density using ImageJ software, highlighting the nature of the relative expression of the given NMD transcripts under pH stress **(Figure 8)**.

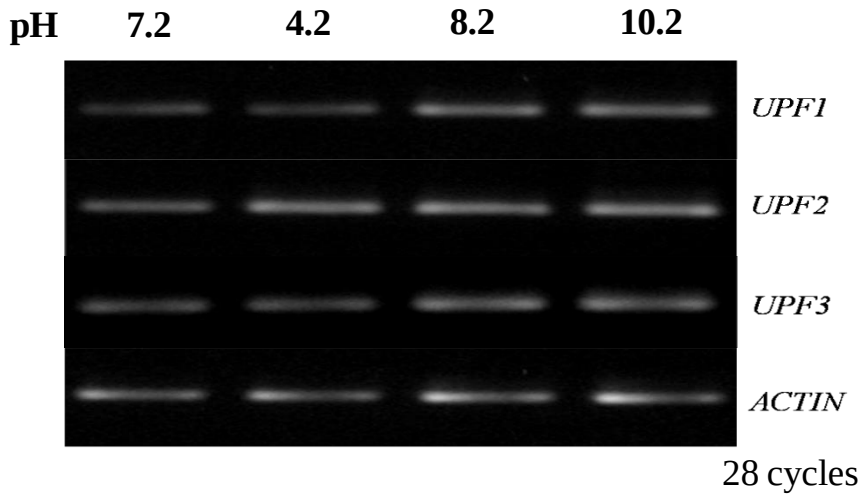

Figure 7. ***RT-PCR and agarose gel electrophoresis showing differential expression of UPFs in response to pH exposure.***

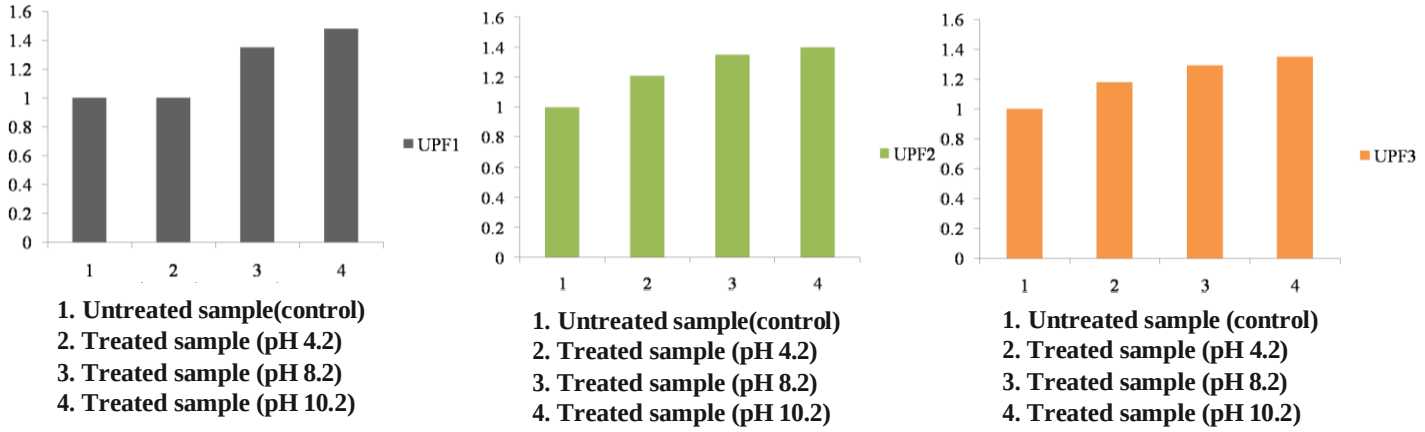

Figure 8. ***Fold- change in the level of UPFs upon exposure to different pH.***

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 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, behavioral changes are seen prominently, along with increasing stress levels. Most importantly, the expression of NMD core factors is altered when the zebrafish was exposed to varied stress conditions, highlighting the effect of pH stress on the NMD surveillance mechanism. This shows that abiotic stress alters the physiological conditions and challenges the organism by altering key molecular signalling events, including NMD, thus leading to the mortality of the zebrafish. Further investigation will shed detailed molecular mechanisms to understand the interlink between abiotic stressors and NMD regulation in eukaryotic model organisms and possibly address the United Nations' sustainable development goals (SDG), such as SDG 14.

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