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Secondary stress responses of zebrafish to different pH: Evaluation in a seasonal manner

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ABSTRACT

Stress is one of the most critical factors in fish health. The response to stress in fish is characterized by the stimulation of the hypothalamus, which results in the activation of the neuroendocrine system and a subsequent cascade of metabolic and physiological changes. The present paper deals with the stress responses of water pH on certain biochemical indices and hematological parameters of zebrafish in a seasonal basis. Zebrafish were exposed to different pH e.g., 7.2, 5.0 and 10.0 and the secondary stress responses were observed. The result showed that exposure to pH 5.0 and 10.0 on zebrafish exerted stresses with reference to seasons. Higher values of blood glucose content observed in the month of summer than the month of winter and females showed higher values than males. Chronic effects of pH on the hematological parameters were also significant between these seasons. In conclusion, exposure to pH at sub-lethal concentrations induced biochemical and hematological alterations in zebrafish and offers a simple tool to evaluate the potential risk of polluted water (acid and base) to fish.

1. Introduction

Stress is any condition that causes physical or mental discomfort that result in the release of stress-related hormones or results in specific physiological responses. Stress responses in fish have been broadly characterized as primary, secondary, and tertiary responses (Goos and Consten, 2002). The primary response is the perception of an altered state by the central nervous system and the release of the stress hormones, cortisol and catecholamines into the bloodstream by the endocrine system. Secondary responses occur as a consequence of the released stress hormones, causing changes in hematological parameters. Tertiary responses are manifest in somatic growth and obtain population level (Terova et al., 2009). Stress can either be short and sudden, or long and chronic. Mild short-term stress has few serious health effects, but long-term stress or severe short-term stress contribute to many of the illnesses which might even lead to death in fish. Fish can only maintain these altered states for a short period of time and then they will adapt or the stress will become chronic.

There are dozens of potential stressors to fish among them pH is a common stressor. Sudden changes in pH can stress or kill aquatic life, even when the changes occur within a pH range they typically tolerate. Aquatic life is unable to adjust to the sudden change and is unable to maintain acid-base and ion regulation and ammonia excretion (Das et al., 2006). pH levels that change abruptly cause acute stress and continually elevated or lowered pH levels can cause chronic stress. Many fish adapt to long-term changes, but there are limits. pH changes of more than 1.5 points below or above recommended levels are going to have a negative effect over time and should never be considered acceptable (Fenner, 2001). High (greater than 10.0) or low (less than 4.5) pH values are unsuitable for most aquatic organisms. Young fish and immature stages of aquatic insects are extremely sensitive to pH levels below 5 and may die at these low pH values. High pH levels (9–14) can harm fish by denaturing cellular membranes.

The zebrafish (*Danio rerio*), a small freshwater teleost is rapidly becoming a popular model species in stress and neuroscience research. The current study will examine how pH stress effects on the secondary responses in a seasonal manner using the zebrafish model. The study of biochemical indices (e.g., blood glucose) and hematological parameters (e.g., RBC, WBC, hematocrit) in fish helps for understand the toxicology and environmental stress (Bhagat and Banerjee, 1985). In many instances the body size along with sex and season which are important factors to bring about changes in blood parameters (Bani and Vayghan, 2011). According to Das et al. (2002) hematological parameters may closely related to the

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response of the animal to the environment. Further the environmental pollutants, disease attack and starvation also alter the blood chemistry (Koeypudsa et al., 2007; Anbalagan et al., 2008; Ramesh and Saravanan, 2008). The pH stress effects in a seasonal manner on secondary responses like blood glucose content and hematological study of fishes are limited. Hence the present paper deals with secondary stress responses of zebrafish in relation to changes in water pH.

2. Materials and methods

Adult wild type zebrafish (*D. rerio*) (male and female) were acclimatized in aerated tap water tanks (pH 7.2–7.4) for 10 days prior to the experiment and a 12 h light/dark cycle. Fish were fed twice a day, 0900 and 1500 h, with commercial dry meal (Krishibid Fish Feed Ltd.) supplemented with live zooplankton (enriched with *Cyclops*, *Daphnia* and *Diaptomus*).

In the first step of experiments tolerance of zebrafish to various pH water levels were determined for a period of 72 h. A range of acidic water concentrations of pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5 were made up with tap water using hydrochloric acid in ten glass tanks ($45 \times 30 \times 30\,cm^3$) having 30 l water capacity. Similarly, a range of basic water concentrations of pH 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, 11.5, and 12.0 were made up with tap water using sodium hydroxide. The pH of tap water was between 7.2–7.4, and 7.2 served as control. The pHs of the media were checked by a portable pH meter (YSI EcoSense pH 100A, USA). Groups of twenty, randomly selected individuals were transferred abruptly to each of the experimental baths equipped with aeration. The pH of the acidic and basic baths were measured twice daily (1000 and 1500 h) and maintained in the range of ±0.1 unit. The survival times of individual fish in the various pH concentrations were recorded. Complete arrests of opercular beats followed by complete loss of response to external stimuli were the symptoms used to conclude that the fish were dead.

In the second step of the experiments blood glucose, hematological and behavioral properties were studied in acidic, basic and control exposure fishes. pH of the water was lowered to pH 5.0 and elevated to pH 10.0 from the control level of 7.2, by the addition of sufficient amount of hydrochloric acid and sodium hydroxide, respectively. In this experiment, thirty adult sized (3.9 ± 0.5 cm) lab acclimated fishes were kept in each pH concentration for 30 days. Three replications were used for each treatment (pH 5.0, 7.2, and 10.0). All the experimental tanks were cleaned regularly, each at 2-day intervals. Prior to water exchange, required stock solution of acid and alkali were added to the tap water to create the desired pH of 5.0 and 10.0. These waters with different pH were used to exchange about 90% water in the corresponding experimental tank with similar pH. The exchange was given slowly to avoid any stress to fishes. Continuous aeration through aerators was maintained throughout the experimental period, to avoid the build-up of any free CO_2 in the experimental tanks. They were fed with zooplankton with commercial fish feed throughout the experimental period.

To obtain blood samples, fish were caught gently in a small scoop net and then quickly taken out from the water and fish were anaesthetized by 5 mg/l of clove oil to avoid any handling stress in order to minimize an error in normal blood values. Blood was obtained from the renal artery by cutting off the tail peduncle. Blood glucose was measured by using a digital blood glucose kit (Stefani et al., 2010). A drop of blood sample was placed on the strips connected to the GLUCOLAB™ Auto-coding blood glucose test meter and results were recorded. The values were expressed in mmol/l. Hematological analyses were carried out by standard methods suggested by Blaxhall and Daisley (1973). To examine red blood cell (RBC) and white blood cell (WBC) counts, collected blood was gently pushed into sterilized eppendorf tubes containing anticoagulant (EDTA) to give a final concentration of 5 mg EDTA per cm^3 blood. Blood samples were mixed gently and counts were made using a Neubauer hemocytometer.

Behavioral changes of the fish were also observed. During this study fish were allowed to move freely and there was no additional incorporation of stress except pH. All experiments were carried out at two months of two different seasons (at January when temperature varied from 8 to 13 °C, a representative of winter season and at July when temperature varied from 28 to 33 °C, a representative of summer season).

Median lethal concentration (LC_{50}) values were calculated from the data obtained in acute toxicity bioassays, by Finney's (1971) method of "probit analysis". The data were subjected to an analysis of variance (ANOVA), followed by comparison of means using Tukey's HSD test to determine significance of each data treatment. In addition, the significance of differences between winter and summer values of RBC and WBC were determined by using "*t* test". Significant differences were indicated by p-values <0.001 and <0.05. All statistical analyses were performed using SPSS16.0.

3. Results

3.1. Behavioral observations at various levels of pH

The lower and upper lethal limits of pH for zebrafish were observed to be those concentrations of hydrochloric acid and sodium hydroxide which caused a pH of 3.0 and 12.0, respectively. The mortality of fishes was recorded within two hours of their exposure to these pH levels. The skin and gills of fish were abundantly covered with mucous; the respiratory epithelium of gills was destroyed at both extremes of pH. Under acute acidic environment, the fish assumed a diagonal position with its head toward the water surface, became sluggish, at times convulsive jerking followed by death. Fishes exposed to lethal basic pH exhibited restlessness, swam rapidly with lashing movements of their tail region. Further, it was noticed that fish which were exposed to pH 3.0 showed initial disturbed swimming movements and appeared pale in color after approximately one hour followed by mortality of about 80% and 70% specimens between 24 and 48 h of exposure at winter month and summer month, respectively. On the other hand, about 90% and 75% fishes died at pH 12.0 within 48 h of exposure at winter month and summer month, respectively. The zebrafish kept at all other pH levels 5.5–9.0 did not show any noticeable behavioral changes during winter or summer.

3.2. Tolerance of zebrafish to various levels of pH

The median lethal toxicity study was performed for the concentration of pH ranging from 2.0 to 12.0. The probit analysis showed that the lethal concentration for 50% mortality of the fishes at 72 h were pH 3.9 and 10.8 in the acidic and basic media, respectively (Fig. 1a and b). Acute acid exposure of zebrafish showed resistance to pH levels from 7.0 down to 5.0 and high to 10.0. Almost no death or about 10% deaths occurred at pH 5.0 or at higher pH levels and pH 9.0 or at lower levels. At very lower pH levels (i.e., pH 3.0 and below) and at very higher (i.e., pH 11.5 and higher) they lost their balance, floated with the bodies turned upside down and died. At pH levels of 4.0 and below and 11.0 and higher they suffered from respiratory distress, markedly shown by the gaping of the mouth, forced expansion of gill opercula and reduction in swimming activity. At pH 5.0 and 10.0 these symptoms lasted for a period of 6 to 12 h, then the fish gradually recovered from the stress, and responded normally throughout the experimental period. However, there was no

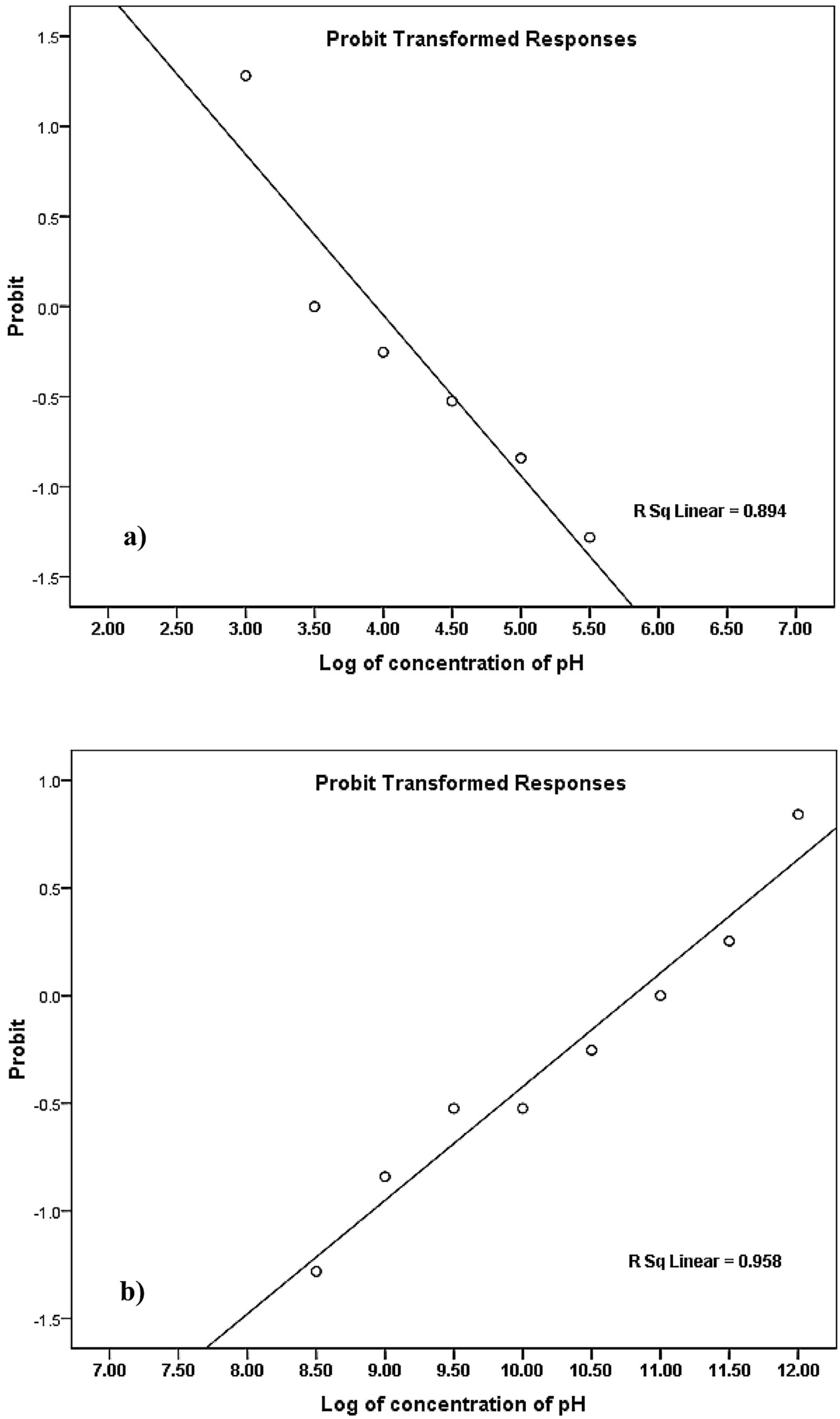

Fig. 1. Probit line graph of acute toxicity of (a) pH 5.0 and (b) pH 10.0 on zebrafish.

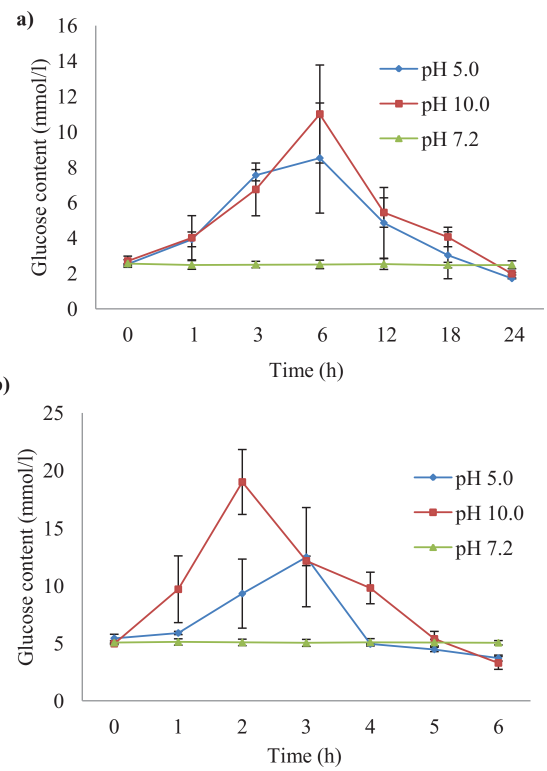

Fig. 2. Zebrafish exposed to different water pH showing fluctuation of blood glucose during (a) winter, and (b) summer. Samples ($n = 5$–7) from individual fish were measured once with each strip. Data are mean and standard deviation. Blood glucose in pH 5.0 and 10.0 are significantly different ($p < 0.05$) from pH 7.2.

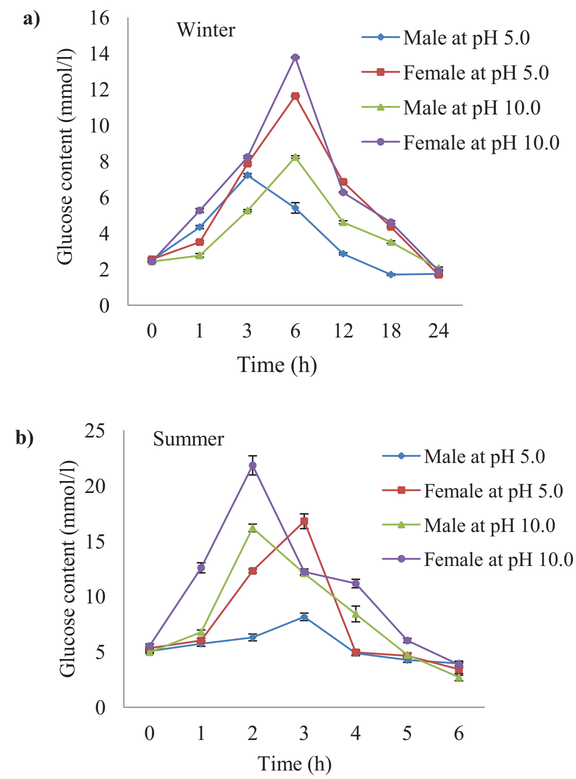

Fig. 3. Male and female zebrafish exposed to different water pH showing fluctuation of blood glucose during (a) winter and (b) summer. Samples ($n = 5$–7) from individual fish were measured once with each strip. Data are mean and standard deviation. Blood glucose in male and female zebrafish in both pH 5.0 and 10.0 are significantly different ($p < 0.05$).

noticeable difference of tolerance of zebrafish to different studied pH observed between winter and summer.

3.3. Glucose level variations on the basis of season

The zebrafish selected for the present research showed variations in the blood glucose level (mmol/l) when exposed to different pH concentrations at two separate months of two different seasons (at January, a peak month of winter season and at July, a peak month of summer season). The zebrafish was exposed to different pH i.e., 5.0, 7.2, and 10.0 and the glucose levels were observed on 0 to 24 h. The amount of glucose in the pH 5.0 and 10.0 exposure fishes were found to be increased within an hour and highest about at 6 h and then gradually decreased during winter season and glucose level get back to its original states within 24 h (Fig. 2a). On the other hand, in summer, the amount of glucose in the pH 5.0 and 10.0 exposure fishes were found to be increased quickly and lasting up to 4 h and then gradually decreased and got back to its original states within 6 to 7 h (Fig. 2b). Overall it was observed that, due to pH stress the glucose level increased in zebrafish up to 24 h period of exposure and rate of increment was higher in summer than winter. In addition, glucose level was much higher in the pH 10.0 exposure fishes than the pH 5.0 exposure. The value obtained by exposure to winter and summer represents the remarkable changes relatively as compare to the control (pH 7.2) (Fig. 2a and b).

3.4. Glucose level variations on the basis of sex

In male zebrafish, blood glucose level varied from 2.04 to 8.23 mmol/l while in female it varied from 1.90 to 13.77 mmol/l of blood at pH 10.0 during winter. Thus higher values were observed in females than males (Fig. 3a). Similar fluctuation pattern was also observed at pH 5.0 during winter but the maximum amount of blood glucose was lower than fish exposed to pH 10.0. During summer, in male, the glucose level varied from 2.72 to 16.18 mmol/l while in female it varied from 3.84 to 21.82 mmol/l at pH 10.0 within 3 h (Fig. 3b). From our result it was also observed that glucose content varied with season. During winter, in female, the maximum amount of blood glucose was 13.76 mmol/l while in summer it was 21.82 mmol/l. Thus higher values were observed in females during summer than winter.

3.5. Variations of the hematological parameters

Table 1 shows the mean values of RBC and WBC counts recorded from exposing zebrafish to different pH concentrations for a period of 30 d. After 7 d exposure, the number of RBC undergone a significant ($p < 0.01$, $p < 0.05$) decrease in both pH (5.0 and 10.0) during winter and summer compared to pH 7.2 (Table 1). Moreover, fish exposed to longer period for 15 and 30 days in both pH had significant ($p < 0.01$, $p < 0.05$) lower number of RBC compared with their counterparts pH 7.2 during summer and winter. Our results also showed that there was a significant difference ($p < 0.05$) of the number of RBC between summer and winter. Results of WBC count showed that there were gradual significant ($p < 0.01$) elevations in both pH groups compared to pH 7.2 group. In addition, like RBC counts there were also significant differences ($p < 0.05$) of WBC counts among pH 5.0 and 10.0 groups and winter and summer values.

4. Discussion

The secondary stress response results from this study on zebrafish that analyzed the water pH, stress indicators in fish, indicated that pH can be stressful to fish. The result of the present study

Table 1
Mean ± SD of the hematological parameters of zebrafish subjected to different pH at winter and summer. $n = 3–5$ for every individual at each pH.

Parameters		Hematological parameters					
		RBC (x10^6 cells/mm^3)			WBC (x10^3 cells/mm^3)		
Season	Day	pH 7.2	pH 5.0	pH 10.0	pH 7.2	pH 5.0	pH 10.0
Winter	0	5.05 ± 0.17	4.97 ± 0.20	4.85 ± 0.38	4.40 ± 0.33	4.29 ± 0.17	4.32 ± 0.19
Summer		5.62 ± 0.03$^{\#}$	5.65 ± 0.07$^{\#}$	5.63 ± 0.03$^{\#}$	4.24 ± 0.17	4.36 ± 0.12	4.34 ± 0.14
Winter	7	4.88 ± 0.26	4.51 ± 0.32*	4.37 ± 0.28*	4.51 ± 0.31$^{\#}$	4.88 ± 0.40*	5.10 ± 0.53$^{*\#}$
Summer		5.60 ± 0.08$^{\#}$	5.02 ± 0.07$^{**\#}$	5.08 ± 0.10$^{**\#}$	4.22 ± 0.11	4.75 ± 0.20**	4.86 ± 0.20**
Winter	15	4.92 ± 0.31	4.09 ± 0.17**	3.73 ± 0.30**	4.33 ± 0.31$^{\#}$	5.89 ± 0.24$^{**\#}$	5.82 ± 0.37$^{**\#}$
Summer		5.59 ± 0.06$^{\#}$	4.36 ± 0.11**	4.16 ± 0.07$^{**\#}$	4.17 ± 0.09	5.62 ± 0.22**	5.57 ± 0.19**
Winter	30	4.82 ± 0.29	3.74 ± 0.20**	3.17 ± 0.16**	4.24 ± 0.54$^{\#}$	6.48 ± 0.34**	7.03 ± 0.33$^{**\#}$
Summer		5.57 ± 0.05$^{\#}$	4.07 ± 0.07$^{**\#}$	4.02 ± 0.09$^{**\#}$	4.10 ± 0.13	6.40 ± 0.21**	6.84 ± 0.19**

Asterisk (*) indicates a significant difference ($p < 0.05$) from control values.
Double Asterisk (**) indicates a significant difference ($p < 0.01$) from control values.
Hash (#) indicates a significant difference ($p < 0.05$) from winter to summer values.

noted that during stress as secondary stress response blood glucose and hematological parameters changed seasonally and this is probably connected with the water quality parameters (e.g., pH, temperature), metabolic activities, gonadal cycle, and feeding intensity.

Behaviour observation is considered a promising tool in toxicity assessments in many species, including fish. Behavioral endpoints serve as valuable tools to distinguish and evaluate effects of exposure to environmental stressors, and fish behavioral alterations can provide important indices for ecosystem assessment (Kane et al., 2005). The most common symptoms of behavioral abnormalities in zebrafish includes imbalance, accelerated respiration, loss of movement and coordination, fish lying at the tank bottom and moving in one spot, subsequent short excitation periods with convulsions and movement in circles, and listlessness before death. Similar observations were also reported by Dobsikova et al. (2006), Khoshbavar-Rostami et al. (2006), and Velisek et al. (2009).

Blood glucose is a highly reliable secondary stress response parameter that is strongly influenced by handling and environmental stresses as well as seasonal variations, nutritional status, and sexual maturity (Prasad and Charles, 2010). The elevation of blood glucose following stress functions to provide energy for the 'fight-or-flight' reaction (Wedemeyer et al., 1990). The lower values in blood glucose level at winter month probably due to low metabolic activities and less intake of food. The lower values during this period are also due to its utilization in the enhanced fat synthesis which accompanies maturation of gonads. The comparatively high level of blood glucose in summer coincides with the favorable temperature of the environment, which occurs mainly due to heavy rain in this region of Indian sub-continent. During this season fish also showed high metabolic activities and more intake of food which results in high level of blood glucose. Glucose content in the blood of zebrafish showed variation in relation to seasons similar trend was reported by earlier workers (Guijarro et al., 2003; Bani and Vayghan, 2011; Sripriya et al., 2012; Suzana et al., 2013) with reference to the differences in glucose level was observed with respect to the development and environment (Das et al., 2002). Blood glucose levels in the females in both seasons were significantly higher than levels in the male determined by this study. This finding may have resulted from the fact that in female zebrafish stored glycogen for egg production leading to increase glucose levels.

In our present study, the change in water pH might have caused the ion regulatory and acid-base disturbances originating at the gill leading to the altered internal pH, electrolyte and osmotic balances. Such disturbances in turn could lead to catecholamine mobilization and disturbances in the RBC homeostasis leading to further activation of the catecholamine induced c-AMP sensitive activation of Na^+/H^+ pump at the RBC membrane surface for efflux of H^+ (Das et al., 2006). Catecholamines were indeed released into the blood circulation as the zebrafish experienced elevated blood glucose levels following exposure to different water pH. The lack of evidence for apical Na^+/H^+ exchange as the predominant acid transporter in freshwater fishes (Nelson et al., 1997) suggests that the zebrafish used an apical vacuolar proton-ATPase and an epithelial ENaC-like channel for the acid secretion and Na^+ absorption, as is hypothesized for freshwater salmonids, tilapia and flounders (Evans et al., 2005). This would ensure extracellular availability of Na^+ for entry into RBCs through Na^+/H^+ exchanger.

Blood offers important profile to study the toxicological impact on animal tissues. Different blood parameters are often subjected to change depending upon stress condition and various other environmental factors (Bani and Vayghan, 2011). In the present study, hematological parameters of zebrafish showed significant variation at different seasons and it was conveniently compared with earlier workers (Anbalagan et al., 2008; Sripriya et al., 2012). The significant decrease in RBC count in the present study might be due to haemolysis and shrinkage of blood cells by the toxic effects of pH. The reduction in the RBC cont of zebrafish following exposure to both acidic and basic pH indicated a reduced blood oxygen carrying capacity (Das et al., 2006). In the context of distortion and lysis of certain RBC cells as observed at pH 5.0 and 10.0, the reduced blood oxygen carrying capacity can be compensated either through increasing oxygen affinity and capacity of hemoglobin and/or increase in RBC production (Das et al., 2006). Though increased production of immature erythrocytes in zebrafish exposed to moderate pH change like those at pH 6.0 (data not shown) and 7.2 explained a possible oxygen compensating mechanism against the stress at moderate pH (Gill et al., 1991), there was a lack of compensation at more extreme pH (pHs 5.0 and 10.0).

In the present investigation, an increased WBC count established leukocytosis, which is considered to be of an adaptive value for the tissue under chemical stress. The increase in WBC count can be correlated with an increase in antibody production which helps in survival and recovery of the fish exposed to toxicants (Joshi et al., 2002). This also helps in the removal of cellular debris of necrosed tissue at a faster rate (John, 2007). As a protective response of the body during stress, WBC increases through stimulation of leukopoietic process and enhanced release of leukocytes to the blood circulation. The released catecholamines, adrenaline and nor-adrenaline, increase the conversion of liver glycogen to blood glucose to satisfy the greater energy demands of the body to stress (Begg and Pankhurst, 2004). The variation of the WBC and increased blood glucose levels in the present study indicated elevated stress levels in the zebrafish which were most likely due to the disturbance in the acid-base balance, respiratory homeostasis and ionic regulation during exposure to the acidic and basic water pH. Consistent supports to the above the several results showed a significant increase in the WBC (Akinrotimi et al., 2012; Far et al., 2012; Geetha, 2014; Wafaa, 2014).

5. Conclusion

The stress response has major role in responding to unpredictable or extreme environmental events, and also has the potential to signal changes in environmental quality. Changes in pH interfere with a number of physiological processes in freshwater fish either by reducing oxygen uptake by the gills or due to changes in the structure of hemoglobin which are accompanied by acid base imbalance. Thus the blood glucose and hematological studies carried out under present investigation showed that zebrafish can tolerate sub-lethal acidic and basic conditions by developing physiological and biochemical adaptations to cope with these constraints. While many of the physiological changes documented in fish during stress still remain within the realm of experimental research and need further study, some have proven useful for quantifying stress in fish resulting from man-made activities, such as water pollution and habitat alteration, and in aquaculture.

Conflict of interests

The authors declare that they have no competing interests. The authors alone are responsible for the content and writing of the paper.

Authors' contributions

MMZ and MSI conceived, designed and performed the experiments described in this work and wrote the manuscript. MMZ, FH, and GMM collected the data. MSI supervised the work and analyzed the results. All authors read and approved the final manuscript.

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