EFFECT OF pH AS A STRESSOR ON THE STRESS RESPONSES IN ZEBRAFISH (Danio rerio) IN ZEBRAFISH (Danio rerio)

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EFFECT OF pH AS A STRESSOR ON THE STRESS RESPONSES IN ZEBRAFISH (*Danio rerio*)

MS Thesis

Md. Mahiuddin Zahangir

Department of Fisheries Biology & Genetics
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Mymensingh

June 2014

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A Thesis

Submitted to
Bangladesh Agricultural University, Mymensingh
In Partial Fulfillment of the Requirements for the Degree of
Master of Science
in

Fisheries Biology & Genetics

By
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Approved as to style and contents by

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Chairman, Defense Committee
and
Head, Department of Fisheries Biology & Genetics

June 2014

Dedicated to
My
Beloved Parents

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June 2014 *The Author*

EFFECT OF pH AS A STRESSOR ON THE STRESS RESPONSES IN ZEBRAFISH (*Danio rerio*)

Md. Mahiuddin Zahangir

ABSTRACT

The experiment was conducted to know the stress responses due to pH effect in zebrafish (*Danio rerio*) over a six month period. The objectives were evaluated by rearing fish species in different pH treatments and periodic sampling of some physiological parameters. During the study it was found that fish can tolerate a wide of pH range from 4.3-10.7. Skin color changed due to long term exposure in different pH treatments. Blood glucose levels varied from 2.53 mmol/l to 7.23 mmol/l in acidic (pH 5.0) treatments and 2.43 mmol/l to 8.23 mmol/l in basic (pH 10.0) treatments. RBC counts showed a significant decrease in acidic and basic treatments while WBC showed a gradual increase compared to control. Hatching rates and hatching time varied from 37-82% and 68-84 hrs, respectively at different treatments. Highest hatching rates and minimum hatching time were at pH 7.0 and lowest hatching rate and maximum time were at pH 4.0. Fish also showed a distinct behavior in swimming, feeding and egg laying at different pH treatments. These results suggest that fishes are highly sensitive to changes in acidity or basicity and they attempt to avoid an environment perceived to be deleterious to their offspring. These parameters are indicative of pH toxicity to fish and may be used as early indicators in toxicity of aquatic animals.

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CHAPTER I

INTRODUCTION

pH is one of the sole contributors affecting the water quality. Acid and bases of pH and other hazardous chemicals in all aquatic water bodies are a common phenomenon due to release of pollutants from the industries in the present day life. The aquatic environment is particularly sensitive to toxic contaminants since a considerable amount of chemicals used in industry, urbanization and agriculture enters in the aquatic environment. At present rapidly growing industrial activities have lead to continuous release of acidic and basic pollutants which may cause damages to the physiology of aquatic animals. Increased pH due to release of chemicals may also cause the destruction of gill tissue and loss of electrolytes from the aquatic organisms. In a word, it affects the physiological process of body.

In the present day of modernization a huge amount of industrial activities are flourishing throughout the world. To maintain such type of trend, Bangladesh is trying to maintain a smart position in the world. Country like Bangladesh has around 42% uneducated people and most of them have no proper knowledge about the basic environmental safety. As a result, they often do some sort of activities which are harmful to the environment especially sensitive to the aquatic environment. Aquatic environment is particularly sensitive to pollution as body physiology of most of the aquatic animals is regulated by water quality criteria.

pH comes to the water body from many sources. These sources either directly or indirectly increase or decrease the water pH in a natural environment along with other pollutants which contain acidic and basic substances. The following are the primary sources which may directly deposit into the open water and increase the water pH.

- Industrial pollution
- Urbanization
- Use of agricultural insecticides and pesticides
- Household garbage
- Small scale industries
- Washing of medical equipment at open water
- Acid rain
- Municipal waste

Mathematically, pH is the negative logarithm of the activity of the (solvated) hydronium ion, more often expressed as the measure of the hydronium ion concentration. In chemistry, pH is a measure of the acidity or basicity of an aqueous solution. Solutions with a pH less than 7 are said to be acidic and solutions with a pH greater than 7 are basic or alkaline. Pure water has a pH very close to 7.

$$\mathrm{pH} = -\log_{10}(a_{\mathrm{H}^+}) = \log_{10}\left(\frac{1}{a_{\mathrm{H}^+}}\right)$$

pH has many adverse effects on the aquatic environment like soil and lake acidification caused high concentration of aluminum, iron and manganese which bind to phosphorous thus reduce the phosphorous concentration which is one of the essential element for plant growth (Hendrey,1982). pH also affects the food sources of the aquatic animals. Copepods and cladocerans are one of the most important zooplankton groups as feed for fish species. A decreasing number of copepods and cladocerans are being found because of significant decrease of pH level (Almer *et al.,* 1978).

"Hydrogen ion toxic syndrome" is a physiological state which is caused by elevated H^+ concentration results in three toxic syndrome like reduced oxygen uptake and transportation in blood; changes in acid-base balance of intracellular and

extracellular fluids and loss of ionoregulation in fish (Wood and McDonald, 1982). This low level pH exposure also causes "hypoxia" through the induction of excess mucus production, destruction of gill tissue, and the alteration of the oxygen carrying capacity of blood pigments (Ultsch, 1978). Acidosis is an acid induced state sometimes occurs in fish due to passive influx of H^+. Acidosis is sufficiently severe in fish and can be the direct causes of death in fish (Packer, 1979).

According to Ultsch and Gros (1979), increase mucus production from a non-convective layer over the secondary lamellae reduces oxygen transfer rates. They also concluded that an increased mucus production and reduction rate of oxygen transport to the blood is also due to the changes in the pH level. Daye and Garside (1977) recorded that the thresold for increase mucus production was 5.2 for acidic pH and production increased with increasing acid stress.

pH also affects the haemoglobin which is a blood protein that has affinity for oxygen to be transported and is strongly pH dependent as because blood pH determines the affinity of haemoglobin for oxygen (Bohr effect) and maintains it's balance in the blood. Because of poor buffering capacity of the blood fish is very much susceptible to acidosis or changes in the acid-base balance of the blood. Fish exposed to low pH had increased haematocrit in the blood (McDonald, 1983; Wood *et al.*, 1988) and their concentrations increased progressively with decreased pH. The concentration of hemoglobin may increase upto 25% in case of adult fish and it can remain elevated over three months (Audet and Wood, 1988). However, increased haemaocrit, blood volume, viscosity and pressure due to exposure to low pH caused cardiovascular failure in fish exposed to acutely toxic pH conditions and is thought to be the principal cause of death in the toxic syndrome (Wood and McDonald, 1982).

Inability to release all or part of the mature eggs from physiological changes prevents the production and maturation of eggs when fish are exposed to acid

stress during oocyte formation and development may cause the reproductive failure in oviparous fish (Peterson *et al.,* 1982). Extensive reduction in quality and number of eggs have also been documented when maturing females were exposed to low pH. Fathead minnow egg production decreases 50% when adults are exposed to pH 6.0 (Craig and Baksi,1977) while desert pupfish egg production fell 60% at pH 5.0 (Lee and Gerking, 1980). Ruby *et al.* (1978) observed that mature oocyte production in flagfish was 80% and 92% lower than the control (pH 6.7) when fish is exposed to pH 6.0 and 4.5, respectively. This is due to restriction in the synthesis and transportation of yolk proteins, low serum calcium and hormone concentrations which are vital to the transportation and deposition of yolk proteins within the oocyte when fish is exposed to the acid conditions (Peterson *et al*., 1982).

The effect of pH on developing embryos is an important component of reproductive success or failure of a species. Embryonic development at low pH may cause abnormal development and reduce egg growth. Kennedy (1980) observed complete reproductive failure of trout eggs at pH 5.8. During embryonic development the incidence of mortality and abnormalities was 78% and 28% higher, respectively from control. Succesful hatching of embryos requires a softening of the egg capsule, the release of a hatching enzyme and the physical rupturing of the outer layer of eggs (Peterson *et al.,* 1980). At pH 5.0 the activity of the hatching enzyme decreases 10% from the normal condition (Peterson *et al.,* 1980). The effect of low pH on the hatching success was also documented by Daye and Garside (1977,1979), Johansson *et al.* (1977) and Peterson *et al.,* (1980). At pH 4.1 approximately 70% of Atlantic salmon and 17% brown trout alevins died as the results of incomplete hatching. The main contributing factor in reducing hatching success is the inhibition of the releasing of hatching enzyme, chorinase .

Excretion of ammonia is very important for normal physiological condition in fishes which is a complicated interaction of carbondioxide, pH, NH_3 and NH_4^+ (Randall and wright, 1989). Under normal condition, the NH_3 diffuses through the gill

epithelium into the mucus and boundary layers, along concentration gradient and is then converted to NH_4. The rate of the conversion decreases with increasing pH and will not proceed if the pH is higher than 9.5. High pH can cause disruption in the ammonia excretion mechanism across the gill epithelium by increasing the pH of the mucus and boundary layers.

Rainbow trout raised at pH 8.0 became acclimated to pH 9.8 and above 9.8 the fish continued to feed but their activity reduced. Mortality was observed at pH 10.2. When pH was increased from 8 to 9.3 the trout had a temporary loss of appetite and an increase from pH 8 to 9.5 resulted in stress and approximately mortality (Murray and Ziebell, 1984).

Behavior is important in feeding, reproduction, migration, pollution avoidance and inter and intra-species competition. The avoidance of high and low pH may be of great imortance in avoiding chronically and acutely toxic pH. Behavioral changes may be an indicator for adaptive mechanism to prevent further stress (Jones *et al*., 1977). Chronic exposure to high and low pH may alter feeding or olfactory responses, resulting reduced growth, reproduction or long-term survival. Jones *et al*. (1977) also observed that, the activity of Arctic char was negatively correlated with plasma protein and glucose concentrations and attraction to food extract was negatively correlated with haematocrit, glucose and cortisol levels and positively correlated with plasma Cl^- concentrations. Figure 1.1 indicates the physiological and behavioral responses to stress at different circumstances. Figure 1.1 also indicates the abnormal hormone secretion, reduced immunity, abnormal gametogenesis and tertiary responses to stress due to pH.

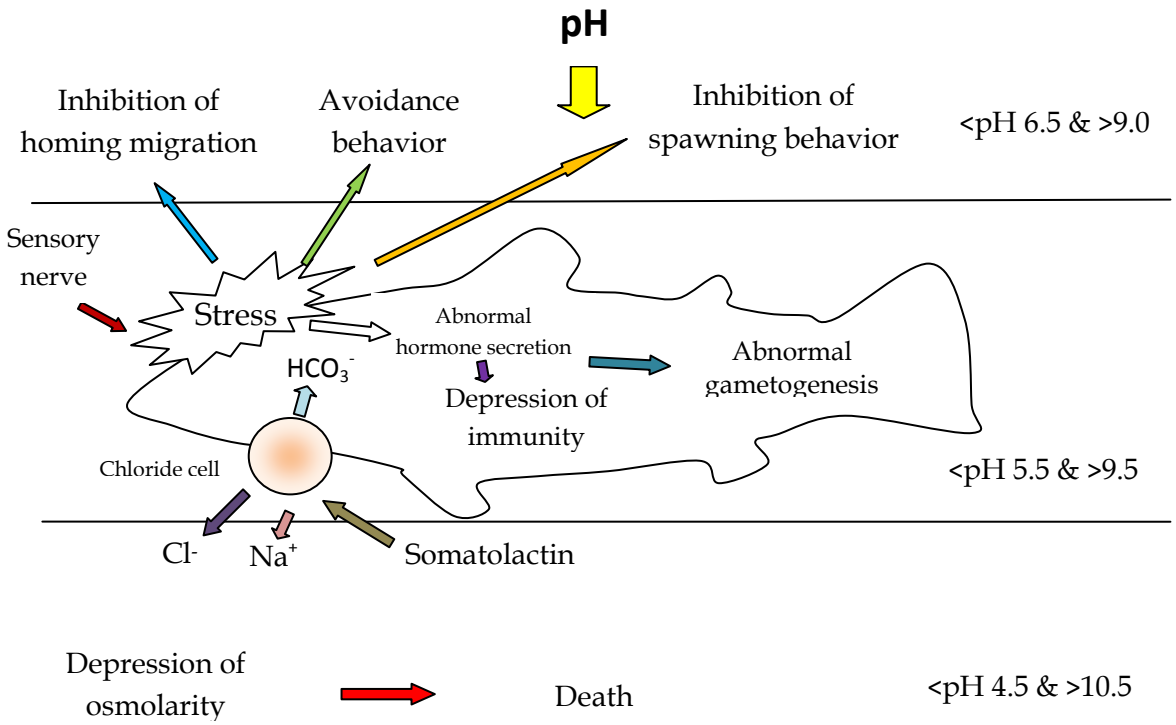

Fig. 1.1 Effect of pH on physiological and behavioral responses in fish (after modification from Ikuta *et al.* 1999)

In recent years the concept of stress as applied to fish has awaked the interest among scientists dedicated to the research of environmental influences on health (Barreto *et al.*, 2006). Physiological or biological stress is an organism's response to a stressor such as an environmental condition or a stimulus. Stress is a body's method of reacting to a challenge. According to the stressful event, the body's way to respond to stress is by sympathetic nervous system activation which results in the fight-or-flight response.

Different stressors stimulate the hypothalamus to release corticotropic releasing hormone (CRH) and vasopressin, which activate the Hypothalamic-Pituitary-Adrenal (HPA) axis. CRH stimulates the anterior pituitary to release corticotropin, which travels through the bloodstream to the adrenal cortex, where corticoptropin upregulates cortisol production. Then the cortisol acts across the entire body to promulgate the stress response (Fig. 1.2).

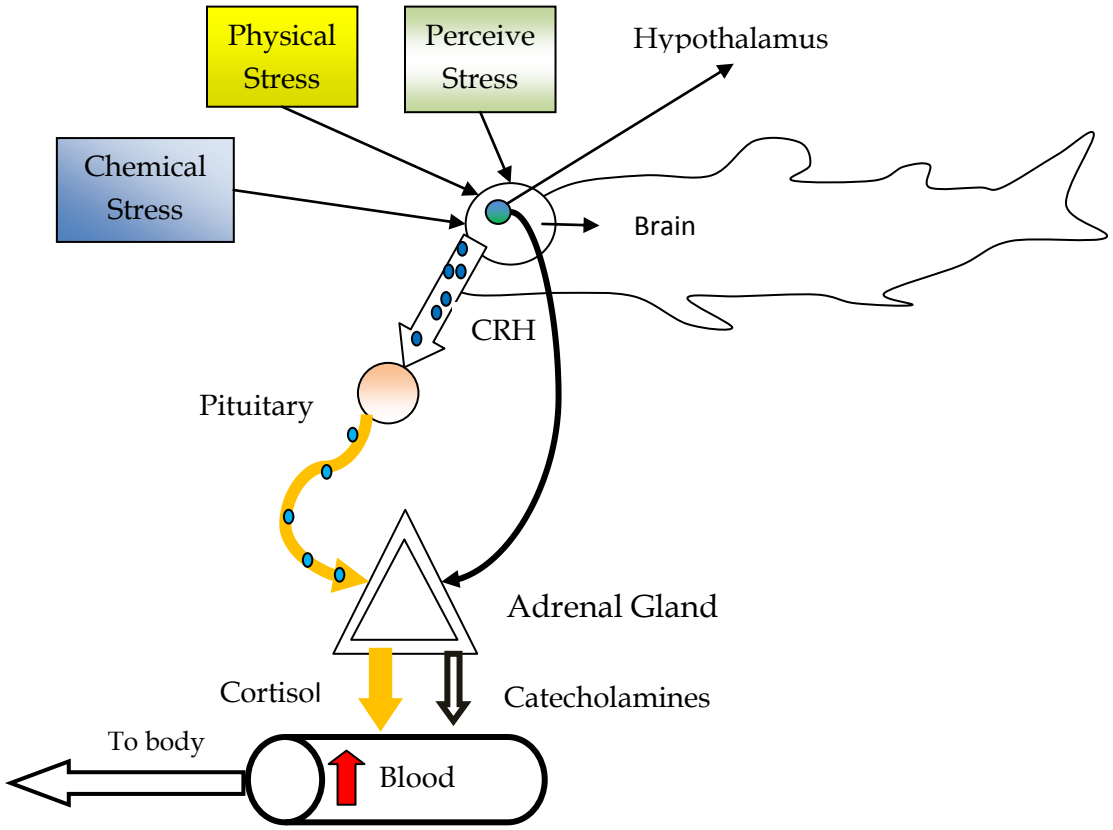

Fig. 1.2 Hypothalamic Pituitary-Adrenal Axis (after modification from Backstrom and Winberg, 2013)

Figure 1.3 shows the physical, chemical and other perceived stressors act on fish to evoke physiological and related effects, which are grouped as primary, secondary and tertiary responses.

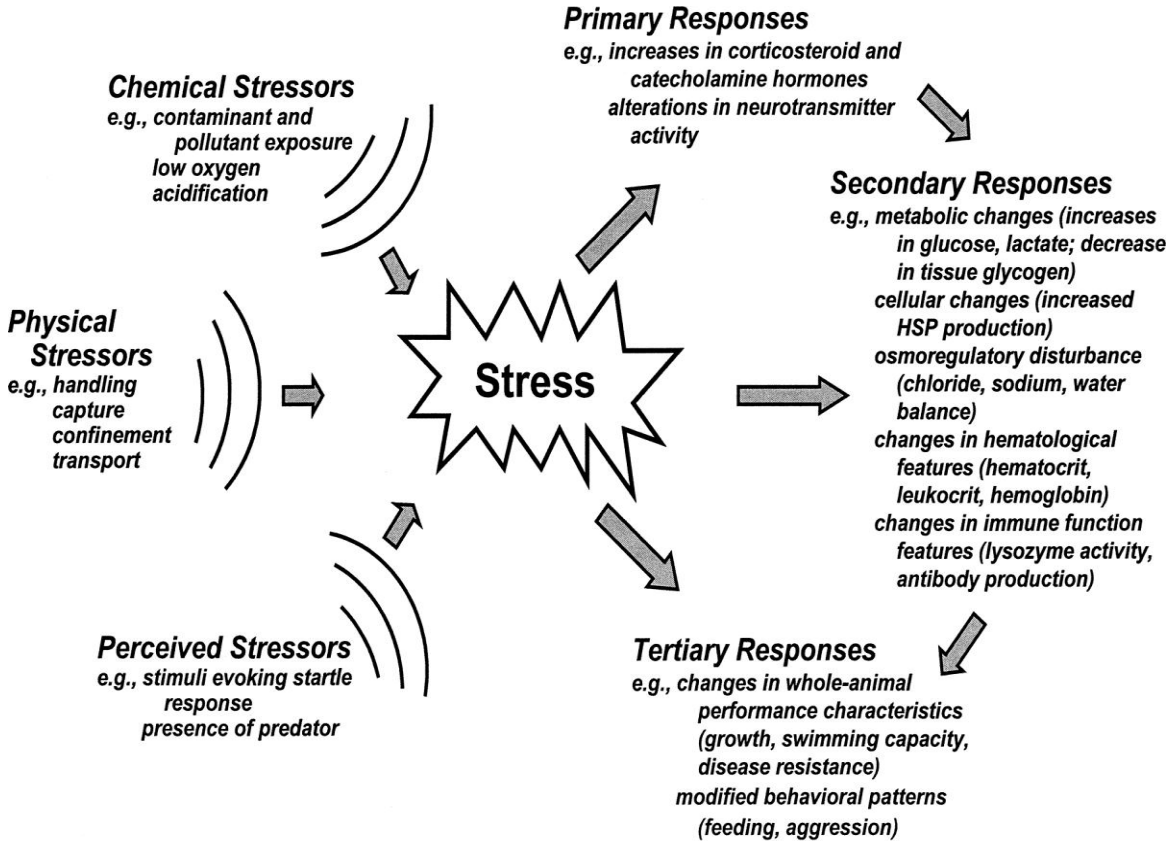

Fig. 1.3 Different stressors and their effects on fish

In order to investigate the effects of pH on general physiology, it is necessary to clarify the biological responses of fish. Therefore, in this experiment the effects of pH on the secondary and tertiary responses were investigated.

Objectives of the research:

1. To examine the effects of acidic and basic pH exposures of acute pH stress on the secondary stress responses (eg., red blood cell, white blood cell, plasma glucose etc.) of *Danio rerio*.
2. To determine the effects of acidic and basic exposures of pH on the behavioral stress responses in *Danio rerio*.

CHAPTER II

REVIEW OF LITERATURE

pH is the negative logarithm of hydrogen ion concentration which has direct and indirect effect on the physiological responses in fish. This chapter is about to review the pH tolerance, blood glucose level, egg collection from different pH medium, embryonic and larval development and changes of hematological parameters due to pH stress in fish. The following information was collected to design the present research and validation of the new findings.

Ekubo and Abowei (2011) reported the pH tolerance of different fish species and determined the acute, chronic and optimum level of pH in different environment. They also reported that, optimum pH range for fish is 7.0 to 8.5. They also compare that pH ranged 7.0 to 8.5 is ideal for biological productivity and pH ranged 4.0 to 6.5 and 9.0 to 11.0 is stressful for fish. Death occurs when pH is less than 4.0 or above 11.0.

Santhosh and Singh (2007) studied the suitable pH range for fish culture is between 6.7 and 9.5 and the ideal pH level is between 7.5 and 8.5 pH above and below these range are stressful to the fishes.

Bhatnagar *et al.* (2004) recommend that, ideal fish pond should have an optimum pH range from 6.5 to 9.0. They also recommended that, pH less than 4.0 and more than 10.5 is lethal to fish/shellfish culture; 7.5-8.5 is highly congenial for *P. monodon*; 7.0-9.0 is acceptable limits and 9.0 -10.5 is sublethal for fish culture.

Serafy and Harrell (1993) conducted a laboratory experiment to show the behavioral responses of fish with increasing pH and concluded that, all three species (Banded killfish *Fundulus diaphanus*, Blugill *Lepomis macrochirus* and Stripped bass *Morone sexatilis*) tested in the laboratory avoid pH when it exceeds 9.5 and it causes the lethal effect for fish species.

Ikuta *et al.* (1992) observed pH tolerance of different fish species and finally concluded that, pH 4.0 constitutes an acidic condition which is lethal for fish species.

Calabrese and Davis (1966) worked on the pH tolerance of clam (*Mercenaria mercenaria*) and oyster (*Crassostrea virginica*) larvae. They reported that the most suitable range of pH for their survival and growth is 6.0 to 9.5. Below 6.0 or above 9.5 there was no growth and survival.

European Inland Fisheries Advisory Commission (EIFAC) (1971) which mainly deals with the water quality indicates that, a safe water pH for fish should be between 6.5 and 8.5.

Timmons *et al.* (2002) noted that most freshwater fish tolerate a wide pH range from 6.0 to 9.5 and it is more practical to maintain a pH range between 7 and 8 for promoting growth and stable water quality for freshwater fish.

Engeszer *et al.* (2007) recorded the presence of zebrafish in waters having pH range between 5.9 and 8.1.

Porchas *et al.* (2009) studied different indicators to measure stress in fish. Much of them were associated to undefined and uncontrolled variables. Some of those factors are related to metabolic changes in the organisms as an adaptation or acclimation mechanism; other is extrinsic to the fishes; other sources of error are caused unconsciously by the researcher during manipulation or due to inadequate control of variables, and may lead to intrinsic changes. Cortisol and Glucose may be useful in acute stress experiments and can easily be monitored throughout time of experimentation. To be used as stress indicator, the physiological status of organism cortisol and glucose are very standardized. They suggested that a reliable source to measure stress in fish body is to measure the cortisol and glucose level of blood.

Raizada and Singh (1982) did an experiment on the blood glucose level in a freshwater fish (*Cirrhinus mrigala*) throughout the year in both male and female fishes and showed that, blood glucose varies seasonally due to temperature changes and it is highest in winter (87.75 mg% in male and 98.97 mg% in female) and lowest in summer (25.22 mg% in male and 27.88 mg% in female).

Bracewell *et al.* (2004) studied glucose level for Chub (*Leuciscus cephalus*) species and recorded the glucose level for 24 hr periods. Plasma glucose levels were significantly higher up to 0.5 hr after simulated electrofishing operations, and peaked (11.0 mmol/l) at 2 hr after treatment. Glucose levels remained high for up to 4 hr.

An experiment was conducted by **Telford (1974)** in crayfish *Orconectes propinquus* and *Cambarus robustus* using artificial stress (handling of fish outside in water) and showed that blood glucose level increases four or fivefold when the crayfish are handled out of water for a period of 2 minutes.

Okomoda and Atagoba (2011) worked on blood glucose responses in *Clarias gariepinus* exposing acute concentration of Glyphosate – Isopropylammonium (Sunsate) for a 96 hr period using static bioassays with continuous aeration under laboratory condition to determine the acute toxicity of glyphosphate in Sunsate on fish. They showed that, blood glucose concentration rises (maximum 6.9±1.1 at highest concentration 20 mg/l) as the concentration of Glyphosate–Isopropylammonium increases. They found a minimum glucose level in controlled experiment (2.9±1.2).

Silver and Shenk (1968) showed a temperature dependence blood glucose concentration in toadfish exposing at four different temperatures in winter (0, 6, 12.5 and 19^0C) and exhibited increasing blood glucose level with decreasing temperature and a disproportionately large rise in blood glucose 9.5mg% and 4.0mg% at temperature 0 and 6^0C which reveals that, blood glucose concentration

increases with the decrease of temperature dependent stress and the level varies at different temperature.

Fast *et al.* (2008) studied the cortisol response and immune-related effects on Atlantic salmon (*Salmo salar*) after exposing to short (a single 15 s out of water) and long-term (4 weeks of daily handling 15 s out of water) stress under culture with *Aeromonas salmonicida* . In the short-term study, samples were collected prior to the application of the stressor and at 1, 3, 6, 12 and 24 hr post stress which reveals that the percentage of free cortisol increased significantly in the stressed group of fish at 1 and 3 hr post stress. Plasma glucose levels were significantly higher than those of control fish at 1, 3 and 6 hr post stress.

Baldisserotto *et al.* (2008) studied the ammonia and pH effect on some metabolic parameters and gill histology of silver catfish (*Rhamdia quelen*) for 96 hr period at different pH (6.0, 7.5 and 8.2) and ammonia (C.I. 0.38–0.49, C.I. 1.25–1.65 and C.I. 1.85–2.36) and showed that, pH affects on muscle glucose, muscle and liver glycogen reduction and also induced gill epithelium damages such as lamellar fusion and edema as compared with controls at different pH along with ammonia in that fish species. Liver glucose and muscle and liver lactate levels increased in all fish exposed to different ammonia and pH compared to the control.

Ikuta *et al.* (1999) reported that, when juvenile carp exposed to a sublethal acidic pH level (4.5), plasma cortisol levels peaked in response to acid stress, and immuno-globulin (IgM) levels subsequently decreased which suggests that acid stress depresses the immune system of fish. They also reported that, mature salmonid fishes when exposed to pH 4.5-5.0; inhibit the development and increases the malformation in their embryos and offspring. Plasma levels of sex steroids and gonadotropin exhibited abnormally high levels and there was a possibility that acid stress disrupted the endocrine control over reproduction. Additionally, the acidic condition of pH 5.8 completely inhibited the homing

migratory behavior of land-locked sockeye salmon, and extremely slight acidification (near pH 6) inhibited their spawning behavior.

Gao *et al.* (2011) experimented on pH effect on fertilization and hatching of Far Eastern Catfish (*Silurus asotus*) at pH ranging from 2 to 13 under laboratory condition and showed that eggs can be fertilized at pH ranges from 3 to 12 and hatching only occurs at pH 4-10 and highest hatching rate (52%) is at pH 7.0. A clear difference in the hatching rate and time was observed at different pH level.

Carrick (1979) worked on acid water effect on the hatching of salmon (*Salmo salar*), sea trout (*Cynoscion nebulosus*) and brown trout (*Salmo trutta*) eggs in the pH of 3.5±7.0 over a period of three winters. The effects of fertilizing the eggs in acid water at pH 4.0, 4.5 and 5.0 was studied and concluded that a considerable yearly variation was observed in the numbers of eggs which are hatched, but there were no marked differences between the three species in the tolerances of the eggs to acid water; pH 3.5 was lethal within 10 days to all the eggs, but at pH 4.5 and higher pH levels there was no obvious difference in hatching attributable to acidity.

Rask (1983) did an experiment on low pH effect on the sensitivity of perch eggs, fry, young of the year, and adult fishes of Perch (*Perca fluviatilis*) and showed that, hatching of eggs in controlled pH (6.4) is always greater (100%) than the acidic pH and hatching rates decrease with the lowering of pH. The eggs were most sensitive soon after fertilization and larvae after hatching while the adult perch were most tolerant. The variation in pH sensitivity of eggs between single female was wide. The pH sensitivity of eggs from two perch populations showed a difference at pH 4.0. The fry became acclimatized to the pH; the lower the pH at hatching, the better tolerance to low pH as fry. At a constant low pH there was a correlation between the conductivity of water and pH sensitivity of eggs.

Trojnar (1977) conducted a study on egg hatchability and tolerance of brook trout (*Salvelinus fontinalis*) fry at sub lethal pH level by incubating eggs at pH 4.6, 5.0, 5.6 and 8.0 and found that, hatchability ranges from 76-91% and highest hatching rates (91%) was at controlled pH (8.0). Differential mortality was experienced when subsequent swim-up fry were exposed to a different pH indicating an acclimation effect.

Craig and Baksi (1977) conducted an experiment on the effects of depressed pH with copper on flagfish reproduction, growth, and survival for a 15 days periods and showed that, flagfish spawning and egg production ceased within four days after acid (pH 5.5-6.8) were introduced with copper, on the other hand control groups continue spawn throughout the exposure period and mean daily output of results were comparable with the experimental study. The copper effect was more pronounced than the pH effect impairing reproduction at concentrations of 7µg/l above a background level of 7.3 ppb.

Vuorinen *et al.* (1999) studied on adult whitefish (*Coregonus wartmanni*) exposing them at a low pH (5.75 or 4.75) for a long period of time (143 days) before and during their spawning season with or without aluminium (150 µg Al/l). Their experiment reveals that, ovulation delayed on average 10 days at low pH. They also showed that, plasma Na^+ and Cl^- concentration was greatly reduced and glucose concentration was significantly elevated at this lower pH in both sexes. Extremely reduced plasma Na^+ and Cl^- concentration, an elevated blood glucose concentration and smallest growth at pH at 4.75 with aluminium were seen in females which suggest that the female reproductive functions made them highly susceptible to alluminium at low pH. In the gill epithelium, deposits of Al were detected in fish exposed to Al and the gill epithelial cells showed hypertrophy at pH 4.75.

Kohanestani *et al.* (2013) worked in Zaringol Stream at Golestan Province, Iran, on seasonal variations in hematological parameters of Spirlin (*Alburnoides eichwaldii*) throughout the year in both male and female fishes and found that hematocrit, hemoglobin, MCHC, red and white blood cells counts showed significant increase in warm seasons comparing with colds and there was no significant effect on haematological profile is dependent on sex except haematocrit. They also concluded that, hematological parameters reflect environmental condition and physiological status of fish.

Gupta *et al*. (2013) worked on seasonal variations (spring, summer, monsoon, autumn and winter) in hematological parameters of Golden Mahseer (*Tor putitora*) and showed that there is a significant seasonal variations in the number of white and red blood cells, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration have been observed. They found higher values of RBC dependent parameters like TEC, Hb, PCV during spring, summer, monsoon and autumn, and lowest during winter. White blood cells on other hand, exhibit gradual decline during monsoon and autumn upto winter only to rise again in spring and summer. They also mentioned that there is a relationship between seasonal changes dependent stress in environmental factors such as temperature and dissolved oxygen with various blood parameters.

Bozorgnia *et al*. (2011) experiment on different acute exposure of temperature (15, 25, 32^0C and controlled temperature) on the blood parameter on common carp following 12hr exposure and showed that red blood cell level increased at 32^0C and decreased at 15^0C when compared to control and had significant different. Hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) in RBC were changed but hematocrit was unchanged at 12 hr. In acute experiment the number of WBC level increased at 32^0C and decreased at 15^0C when to compared to

control. They also suggested that temperature and time of exposure influence blood parameters of *Cyprinus carpio*.

An experiment was conducted by **Adeyemo (2007)** on *Clarias gariepinus* after exposing to lead stress and studied on the blood parameter for a 96 hr at different concentration (0, 25, 50, 100 and 200 mg/l of lead nitrate) and showed that, the Packed Cell Volume (PCV) decreased significantly relative to that of the control, while their platelet counts increased compared with the control. There was also a reduction in the RBC in blood of treated fish. Other blood parameters did not vary significantly in comparison to the control group, but the Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) increased considerably in all treatments compared to the control. He concluded that, direct or feedback changes in structure of RBC resulting in haemolysis and impairment in haemoglobin synthesis, stress related release of RBCs from the spleen and hypoxia, which was induced by exposure to lead as a stress.

Radoslav *et al.* (2013) conducted experiment on the red blood cell parameters of *Barbus balcanicus* exposing short duration (60 min) thermal stress (temperature increases from 19 ^{o}C to 29 ^{o}C) effect and showed that there is an increase in Mean Corpuscular Volume (MCV) with a resulting increase in Packed Cell Volume (PCV) and a decrease in haemoglobin concentration in a liter of erythrocytes Mean Corpuscular Haemoglobin Concentration (MCHC), decrease in MCHC values can be related to increase in erythrocyte volume (MCV) which is not followed with adequate increase of haemoglobin in them Mean Corpuscular Haemoglobin (MCH).

Chezhian *et al.* (2009) worked on effects of chemical released from industrial effluent having an undesirable limits of concentration of water quality parameter including pH exposing at different effluent concentration (15%, 20% and 25%) and

observed swelling, hyperplasia, hypertrophy and proliferation of chloride cells of fish exposed to 15% concentration; but in 20% concentration lifting up of the epithelium lamellar fusions and necrosis were seen. Whereas in the gills of 25% effluent treated fish disintegration of epithelial cells, desquamated epithelium, haemorrhage and complete damage of epithelial cells of lamellae of common carp (*Cyprinus carpio* Var. *communis*) were observed.

CHAPTER III

MATERIALS AND METHODS

This chapter deals with the methods that are followed and materials that are used to achieve the objectives of the study. Methodology is an indispensable and integral part of any research. In a scientific research the acceptability of the results depends to a great intent on the appropriate methodology. The results may be erroneous for the use of imperfect methodology. In this study a scientific and logical methodology has been followed by the researcher. The present study was aimed to determine the pH stress effects on the physiological stress responses in fish. This study is based on laboratory work and data are collected for the interpretation of results.

3.1 Study Area:

The experiment was conducted in the Backyard laboratory of the Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh. This experiment was conducted from 1st April to 31st September in the year of 2013.

3.2 Preparation of Aquarium:

A total of eight glass aquaria (45×30×30 cm^3) sized were made locally for this experiment. Each of the aquaria has 30 liter water capacity. Two layers of marble in a petridis were placed as a substrate and artificial trees were also provided in each aquarium for the breeding purposes. Aerator with air stones were also provided for aeration in the aquarium. A thermostat water heater (PERIHA, Aqua Heater, China) was set for controlling temperature.

3.3 Selection of Fish Species:

For this experiment zebrafish (*Danio rerio*) was selected which is locally called "Fuldarkina" or "Onju" and has high research value. Zebrafish was collected from

the field laboratory complex of the Faculty of Fisheries, Bangladesh Agricultural University. This species has been chosen for the following reasons-

- Its genome has been fully sequenced, and it has well-understood, easily observable and testable developmental behaviors.
- Its embryonic development is very rapid, and its embryos are relatively large, robust, and transparent, and able to develop outside their mother (Dahm, 2006).
- The species is nearly constant size during early development, which facilitates simple staining techniques.
- Its two-celled embryo can be fused into a single cell to create a homozygous embryo.
- The zebrafish is also demonstrably similar to mammalian models and humans in toxicity testing, and exhibits a diurnal sleep cycle with similarities to mammalian sleep behavior (Jones, 2007).

3.4 Feeds and Feeding:

Fish were fed twice a day with a commercial floating feed (Krishibid Fish Feed Ltd.) and often with zooplankton (enriched with *Cyclops*, *Daphnia*, *Cladocerans* etc.).

3.5 Water Quality Parameters:

Sl No.	Parameter	Ranges	Device used
1.	pH	5.0-6.0 for acidic, 9.0-10.0 for basic and 6.8-8.2 for control treatments	Portable pH meter (HANNA, HI98107 ROMANIA)
2.	Temperature	27-32^{o}C	Normal thermometer

3.	Dissolved oxygen	3.9-8.5 mg/l	Portable DO meter (YSI, DO200A, USA)
4.	Ammonia	0.1-0.3 ppm	Ammonia test kit (Freshwater) (HANNA,HI 3824 ROMANIA)

3.6 Acid and Base:

Acetic acid (CH_3COOH) and Sodium hydroxide (NaOH) were used to maintain the pH in the aquarium. Acetic acid is a biological acid which pH itself 2.9 and Sodium hydroxide is a strong base and it's pH is 13.0. These two chemicals were chosen because of their availability and small amount was required for adjusting pH in the aquarium.

3.7 Experimental Design:

Fish were reared for a period of six months shown in Figure 3.1:

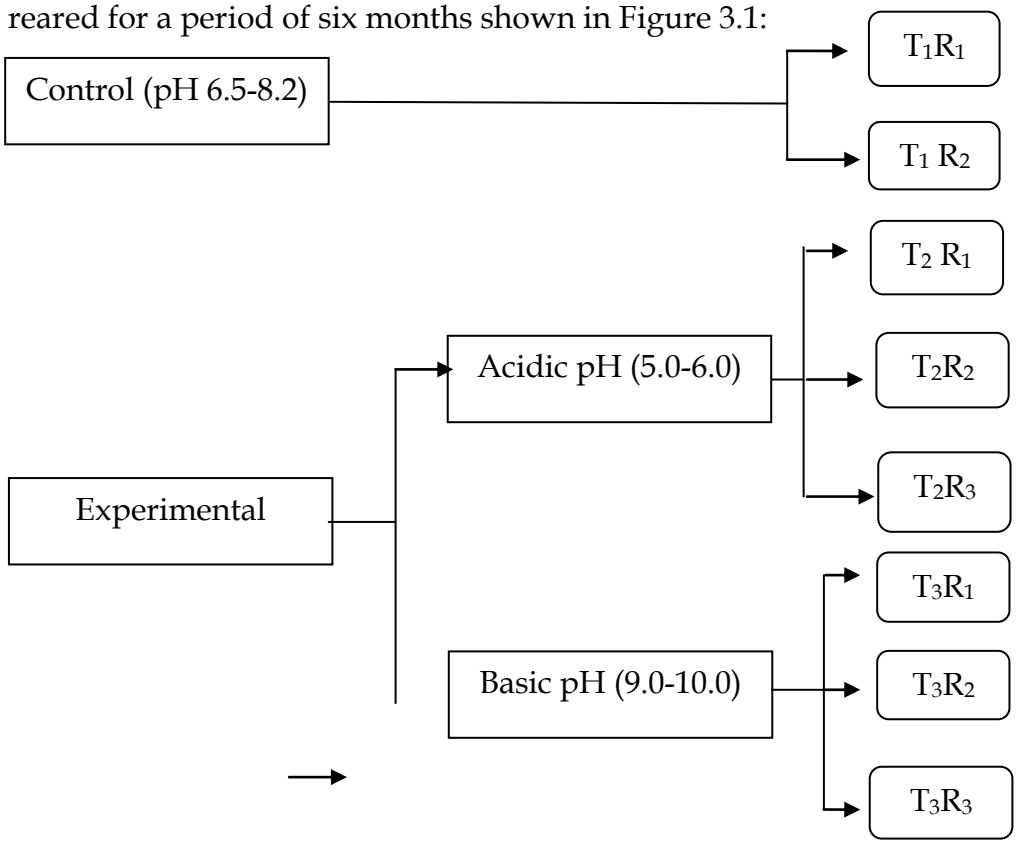

Fig. 3.1 Experimental design

3.8 Measurement of Blood Glucose:

Glucose level of blood was measured by using a digital glucometer, Glucolab (INFOPIA G22A12K2200344, KOREA). Glucose level was measured by cutting the tail of fish by sacrificing the species. At first, a strip of the glucometer was inserted into the meter and then about 2 µl blood samples were touched in the specific position of the strip and the data were recorded as mmol/l (Fig. 3.2).

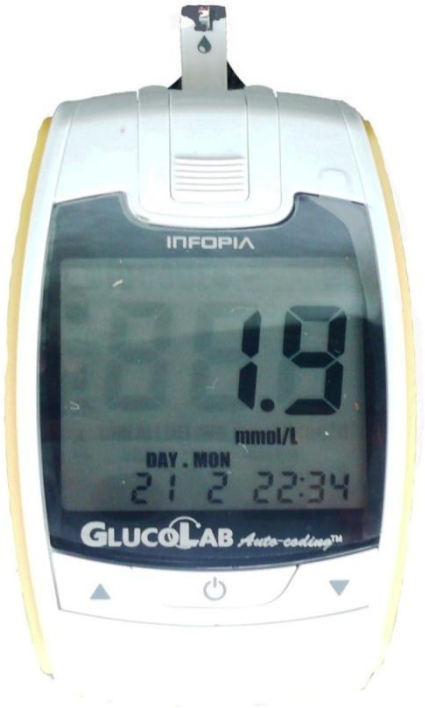

Fig. 3.2 Estimation of glucose in the blood

3.9 Collection of Blood:

Blood samples were collected by cutting the caudal peduncle area of fish and it blood was collected with the help of a micropipette (Fig. 3.3). Before taking the blood, micropipette was rinsed with 1N EDTA solution to avoid the clotting of blood. 5 µl of blood and 45 µl EDTA (2%) solution was taken in an eppendorf making 10% concentration of blood for further hematological study.

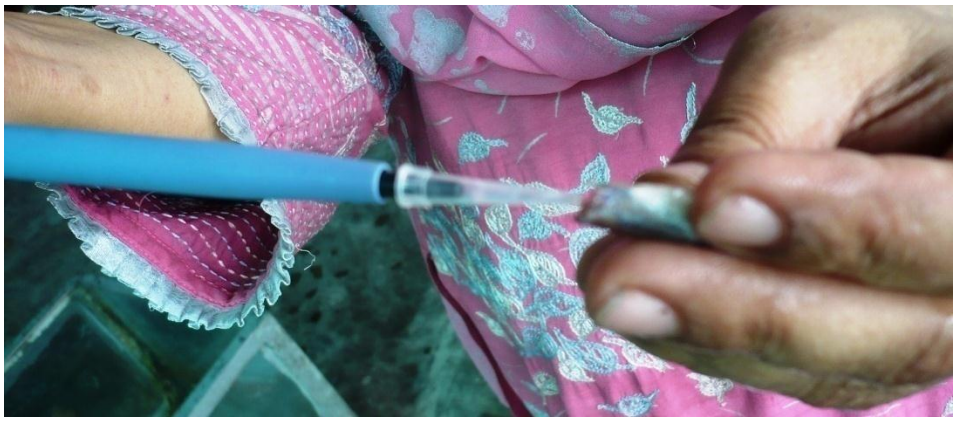

Fig. 3.3 Collection of blood

3.10 Counting of Red Blood Cell (RBC) and White Blood Cell (WBC):

Counting of red blood cell and white blood cell was also done for this study. Blood cells were counted by using a Haemacytometer (Superior, GERMANY). At first a cover slip was placed on the counting chamber of haemacytometer. Small amounts of known concentration of blood were spread out in between the gaps of cover slip and the haemacytometer. Then the slide was gently transferred and carefully placed under the microscope for counting. Counting of cells was done by observing the cells on haemacytometer and randomly counted at least 5 smaller units of haemacytometer. Records of the count were kept and results were calculated by the following formula:

$$\frac{\text{Number of cells counted} * \text{dilution} * 4000}{\text{Number of smallest square counted}} \text{ Cells/mm}^3$$

3.11 Collection of Egg:

Egg collection is very important for any embryological study. In the present study eggs were collected at 8 AM daily from the aquarium. Eggs were deposited in between the gaps of marble of the petri dish. To collect the eggs petri dish was taken out from the aquarium and the eggs were separated from the marble transferred into a hatching tray. Embryonic developmental stages were observed

with the help of a microscope equipped with a camera (OPTICA B350, ITALY). The egg collection method is shown in the flow diagram (Fig. 3.4).

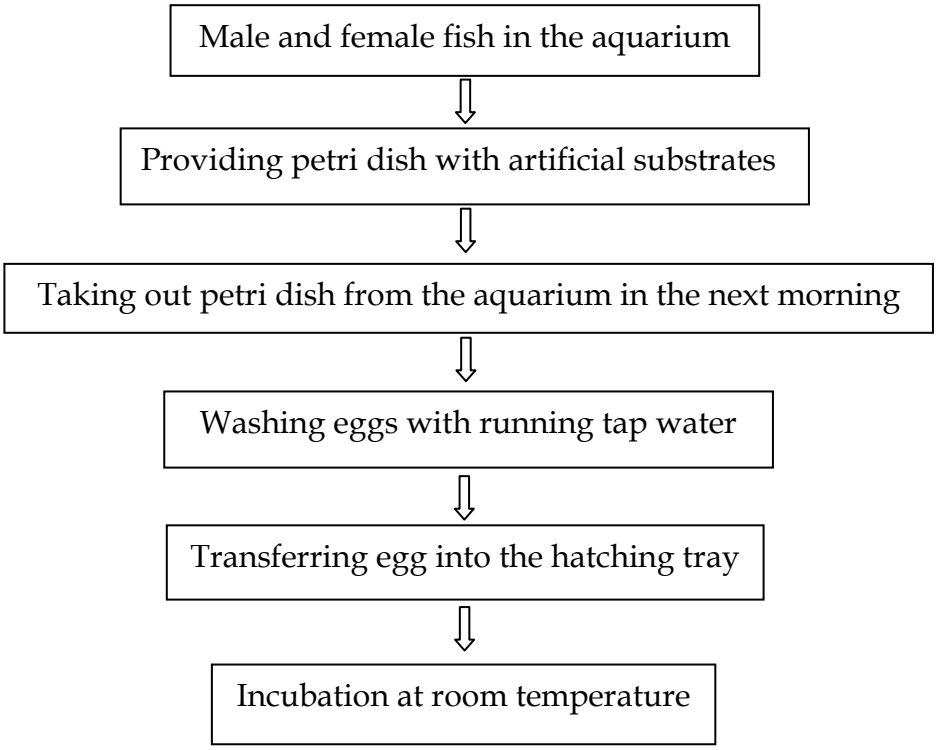

Fig. 3.4 Egg collection method from aquarium

3.12 Examination of Egg:

Eggs were kept in a hatching tray at different pH media. Embryonic developmental stages were studied under a camera attached microscope and photographs were taken using the camera equipped with the microscope (Fig. 3.5).

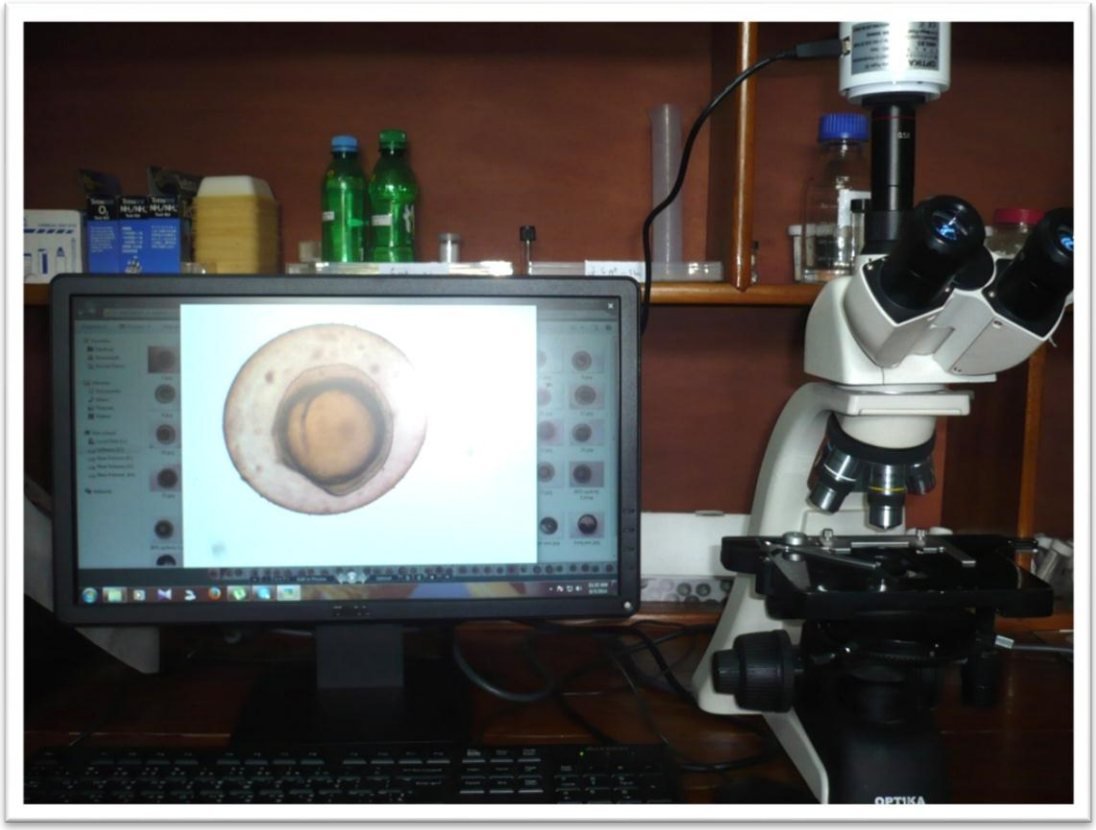

Fig. 3.5 Observation of embryonic developmental stages

3.13 Behavioral Study:

Behavioral study of zebrafish was done at different pH by naked eye. During behavioral study fish were allowed to move freely and there was no additional incorporation of stress except pH. This study was conducted at different pH level and when acid and base were incorporated to the aquarium to adjust pH. A SONY DSC-W220 camera was used to keep the records of video and still images of the fish behavior.

CHAPTER IV

RESULTS

This chapter is basically a simple descriptive part of the study dealing with the outcomes of effect of pH on the physiological stress responses in zebrafish. Result is an important part of any research work. From the present experiment, detailed information about pH tolerance, embryonic development, hematological profiles were presented in this chapter.

4.1 pH Tolerance:

Zebrafish is a hardy fish species. This species can tolerate a wide range of pH from 4.3 to 10.7. Below 4.3 and above 10.7 pH became lethal for this species. When pH was below 4.3 or above 10.7 this species began to suffocate and died within a short period of time. pH 11.0 or more and 4.0 or less were completely lethal for zebrafish. Sublethal dosages of zebrafish were 5.0 to 6.0 for acidic condition and 9.0 to 10.0 for basic condition.

4.2 Secondary Stress Responses:

4.2.1 Blood Glucose Level

Blood glucose level of zebrafish was recorded in both acidic pH (5.0) and basic pH (10.0) stress for 24 hr. Glucose level varied from 2.53 mmol/l to 7.23 mmol/l in acidic treatments and 2.43 mmol/l to 8.23 mmol/l in basic treatments with a highest peak at 6 hr in both media whereas in the control medium it remained same throughout the experimental period (Fig. 4.1 and 4.2). There was no significant difference among the observed values in both the treatments. Glucose level remained elevated from 1 hr to 12 hr and then got back to it's original states. But significant difference ($P<0.05$) among the values were observed at certain hours (1 hr, 6 hr, 12 hr and 24 hr) in different treatments (Fig. 4.3).

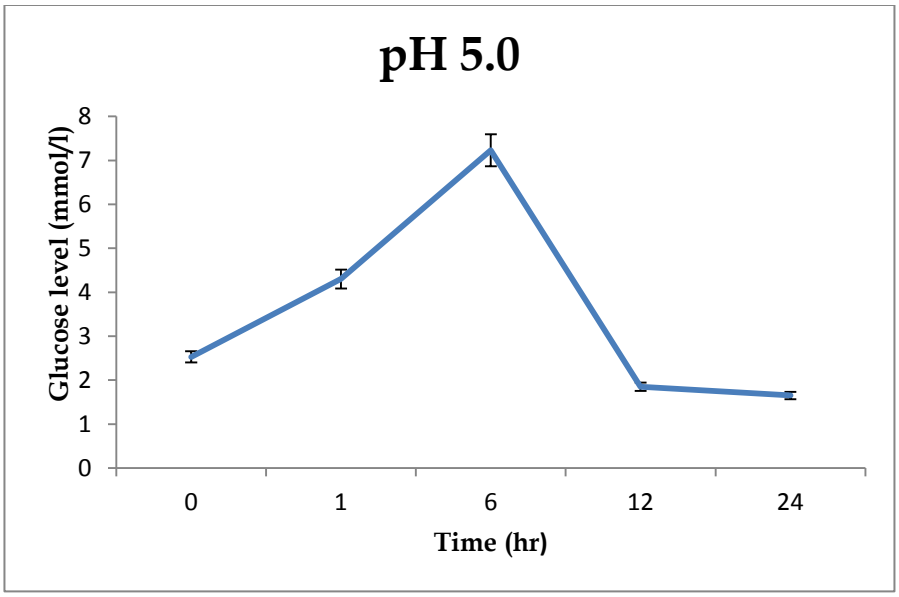

Fig. 4.1 Glucose levels in zebrafish exposed to the sublethal acidic pH (5.0) for 24hr

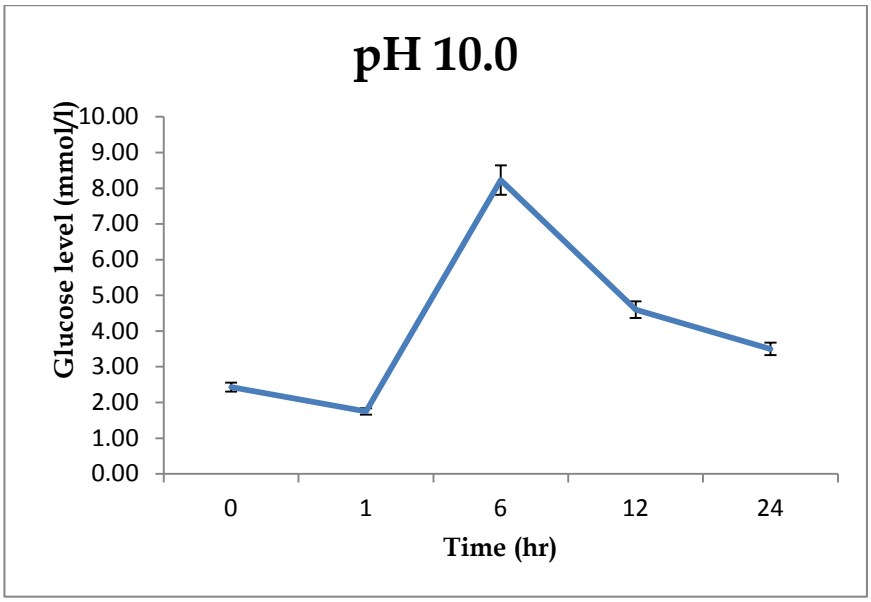

Fig. 4.2 Glucose levels in zebrafish exposed to the sublethal basic pH (10.0) for 24hr

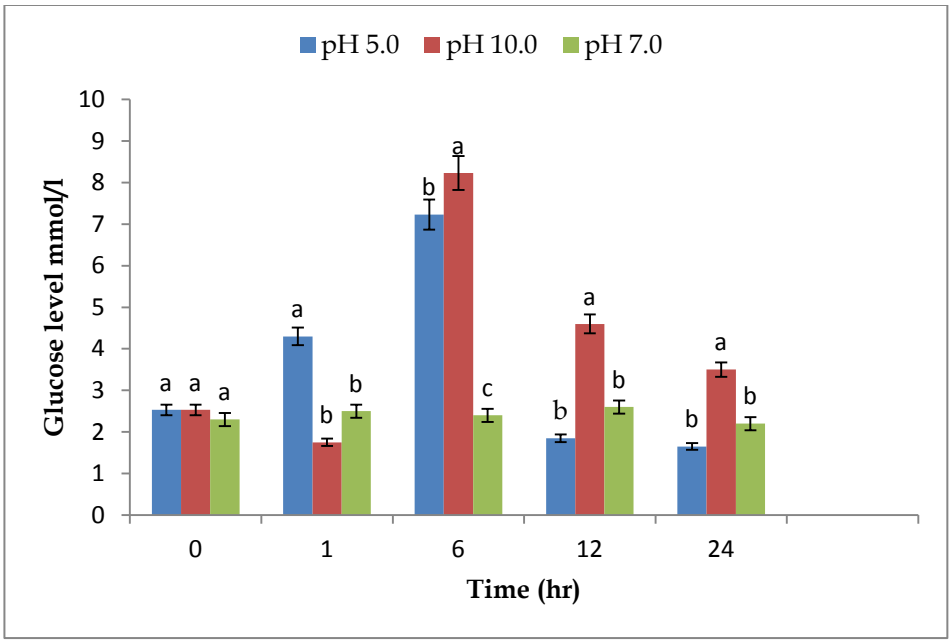

Fig. 4.3 Comparative level responses in zebrafish for 24hr period. Values are presented as mean ± SE. Different superscripts in each treatment group are significantly different at P<0.05.

4.2.2 Counting of Red Blood Cell (RBC)

Number of Red blood cell (RBC) also varied from experimental to control. RBC decreased from 5.68×10^5 cells/mm^3 to 3.21×10^5 cells/mm^3 in acidic treatment and 5.63×10^5 cells/mm^3 to 2.61×10^5 cells/mm^3 in basic treatments. In control pH it remained more or less same throughout the experimental period. The observed values were statistically different (P<0.01) (Fig. 4.4).

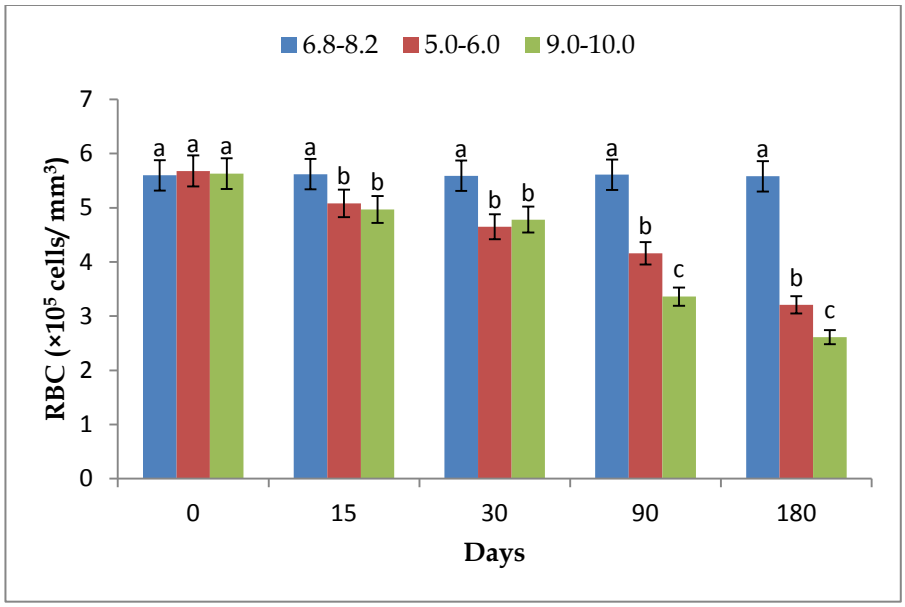

Fig. 4.4 Number of RBCs at different treatments during study period. Values are presented as mean ± SE. Different superscripts in each treatment group are significantly different at P<0.01.

4.2.3 Counting of White Blood Cell (WBC)

White blood cell (WBC) also varied from experimental to control. WBC increased from 4.39×10^3 cells/mm^3 to 7.84×10^3 cells/mm^3 in acidic treatments and 4.34×10^3 cells/mm^3 to 8.53×10^3 cells/mm^3 in basic treatments. While in control treatments it remained more or less similar throughout the experimental period. In this experiment, counting of WBC of fish were studied in between treatments, had a statistically significant difference (P<0.05) (Fig. 4.5).

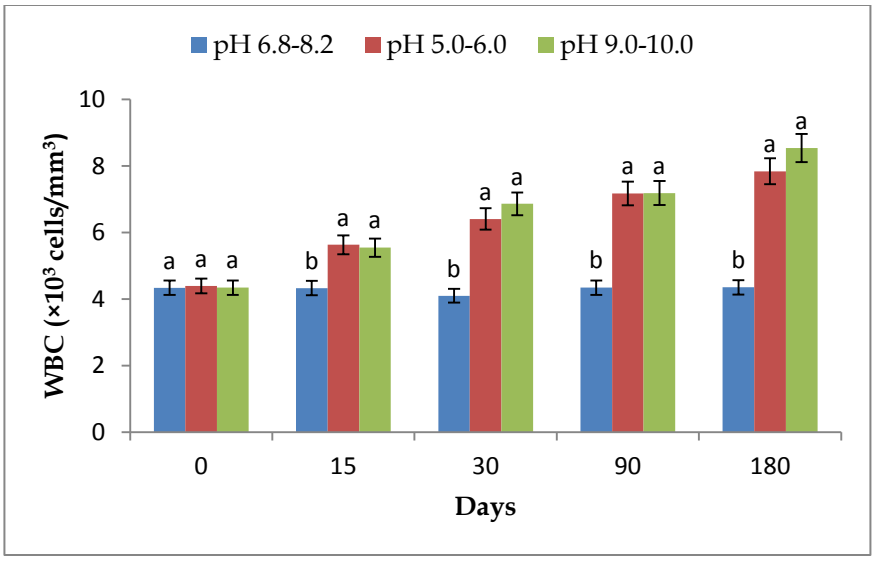

Fig. 4.5 Number of WBC at different treatments during study period. Values are presented as mean ± SE. Different superscripts in each treatment group are significantly different at P<0.01.

4.3 Collection of Eggs:

The frequency of egg collection varied in different treatments. No egg was found in the basic treatment. Eggs were only collected from the control and acidic treatments. Eggs collected from the control group varied significantly (P<0.05) from the acidic treatments. Number of eggs collected from control group was 35±6 while in acidic medium the number of eggs was 16±4. Significant reduction (P<0.01) of the egg deposition was also observed in the alternate day in acidic medium. In that day, the number of eggs reduced to 3±2 or even zeros (Fig. 4.6). Statistically similar results were obtained in control whereas significant difference (P<0.05) was observed in egg collection in acidic medium during the study period.

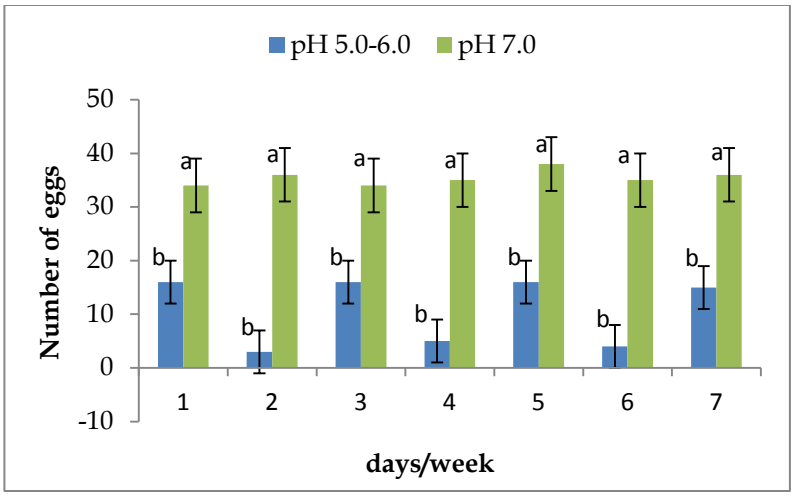

Fig. 4.6 Frequency of egg deposition in the control (pH 7.0) and acidic treatments (pH 5.0-6.0). Values are presented as mean ± SE. Different superscripts are significantly different at P<0.05.

4.3.1 Hatching Rates

Hatching rates of the egg was examined at different pH levels (2.0-12.0). Hatching of eggs occurred between pH 4.0 to 10.0 and the highest hatching rate (82%) was found at pH 7.0. But no egg hatched at pH 2.0 and 12.0. About 37% hatching rate was observed at pH 4.0 and 72% at pH 8.0. Increase or decrease of pH from 7.0 reduced the hatching rates (Fig. 4.7).

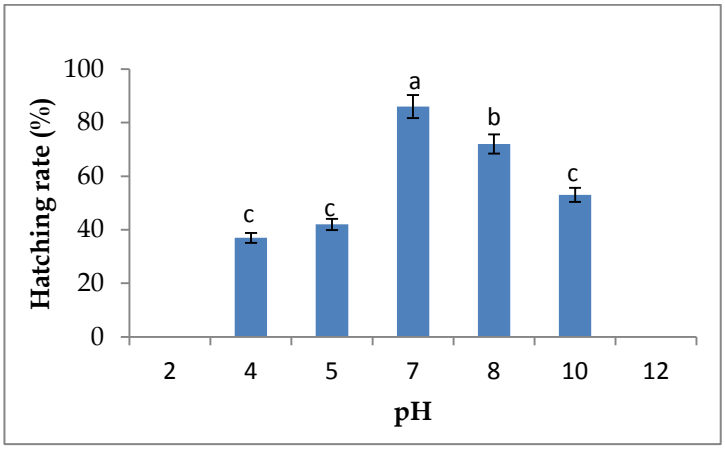

Fig. 4.7 Hatching of egg at different pH. Values are presented as mean ± SE. Different superscripts in treatments are significantly different at P<0.05.

4.3.2 Hatching Time

Hatching rates also varied with time at different pH. In this experiment, it was found that required hatching time varied from 68 to 84 hr. Minimum hatching time was required at pH 7.0 while prolonged time was required to hatch at the above or below pH of 7.0 (Fig. 4.8). Although eggs did not hatch at pH 2.0 and 12.0, their embryonic development continued up to 12 hr and 24 hr, respectively.

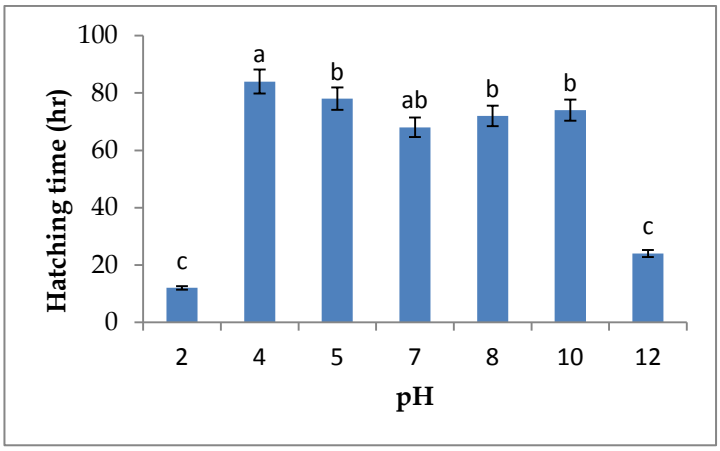

Fig. 4.8 Hatching time at different pH. Values are presented as mean ± SE. Different superscripts are significantly different at $P<0.05$.

4.4 Behavioral Stress Responses to pH:

4.4.1 Behavioral Responses in Normal pH

Zebrafish showed normal behavior by demonstrating normal swimming throughout the water column and shoal formation in a wide range of pH from 6.5-8.0. They also showed frequents feeding, repeated movement and normal metabolism. They did not show any abnormal behavior like aggregation at any corner of the aquarium and did not try to escape from the aquarium (Fig. 4.9).

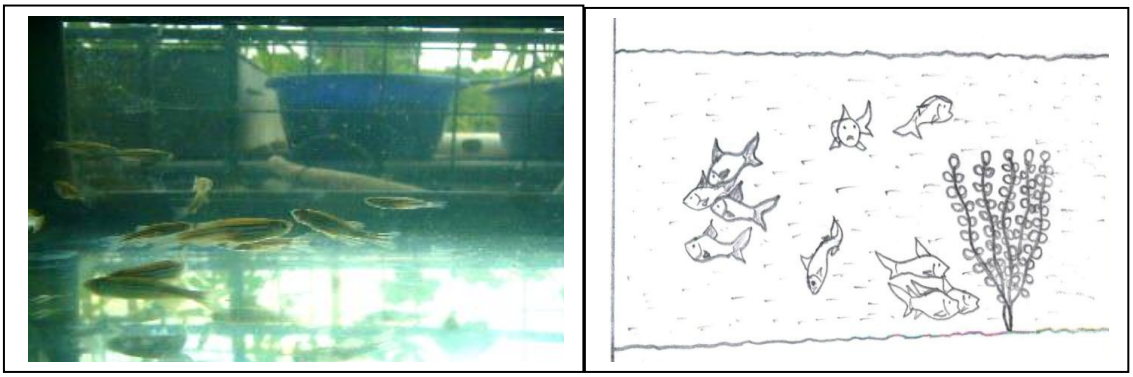

Fig. 4.9 Fish in normal pH medium with normal swimming activity

4.4.2 Behavioral Responses in Acutely Stressed pH

Behavioral responses in acutely stressed condition were much more severe than any other responses. When fishes were in acute stressed condition (sudden changes of pH more than 10.0 or less than 5.0) they did not take any food, movements were increased and sometimes they tried to escape by jumping over the aquarium. Failure of acclimatization with the acute stress, turned them into exhausted and finally fishes were died (Fig. 4.11).

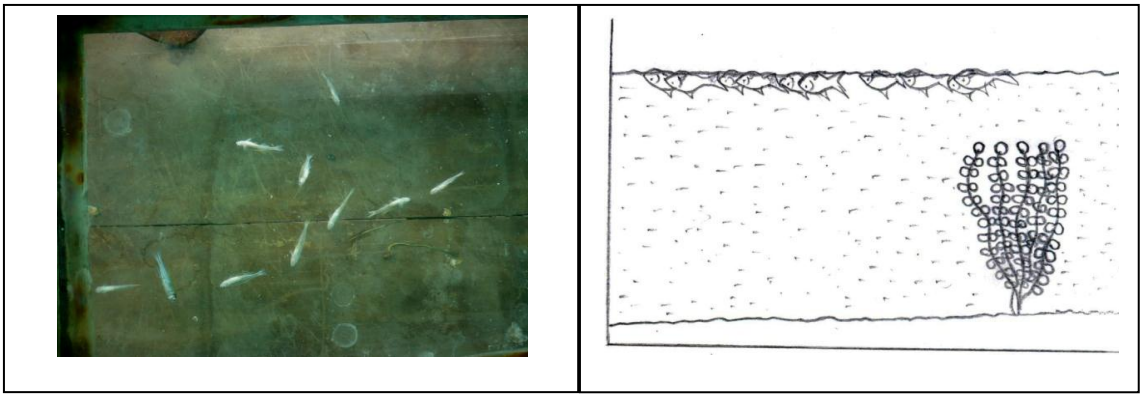

Fig. 4.10 Behavioral responses in acutely stressed pH

4.5 Effects of Sub Lethal pH:

4.5.1 Skin Color

Zebrafish is an ornamental fish. It has a shinning skin and zebra type stripes in their body. Skin color of zebrafish was changed after long term pH exposure. In this experiment, after a long term (6 month) acidic or basic exposure, the skin became dull, gloominess and loss their bright color (Fig. 4.11).

(a) Skin coloration at normal pH

(b) Skin coloration after sublethal basic pH exposure (9.0-10.0) for six months

(c) Skin coloration after sublethal acidic pH exposure (5.0-6.0) for six months

Fig. 4.11 Effect of sublethal pH exposure on skin coloration of zebrafish

CHAPTER V

DISCUSSION

This chapter described the overall results comparison, achievement of the study and problems associated to the research work on physiological stress responses. The objectives of the study were examined the effects of acid and base exposure on the physiological stress responses in their secondary and tertiary levels.

5.1 pH tolerance:

In the present study it was observed that zebrafish (*Danio rerio*) can tolerate a wide range of pH from 4.3 to 10.7. Below or above this range causes lethal effect on it. Zebrafish can continue their normal physiological functions at pH 5.0-10.0 as because it is a hardy fish. pH 5.0-6.0 and pH 9.0-10.0, treated as acidic and basic conditions respectively, were considered as sublethal states for this species.

In a similar study Ekubo and Abowei (2011) reported that pH ranges from 4.0 to 6.5 in acidic condition and 9.0 to 11.0 in basic condition are stressful for fish. Death occurred when pH is less than 4.0 or above 11.0. Bhatnagar *et al.* (2004) recommended that pH less than 4.0 and more than 10.5 are lethal to fish/shellfish culture and 9.0-10.5 is sublethal for fish culture which agreed with the findings of the present study.

5.2 Blood glucose level:

Plasma glucose levels in fishes increase during stress probably as a result of catecholamine action on stored glycogen in liver and other tissues (Pottinger, 1998). In the present study, blood glucose level was significantly elevated up to 6h in the experimental group compared to control. The elevation of blood glucose following stress functions to provide energy for the 'fight-or-flight' reaction (Wedemeyer *et al.,* 1990). In this study, plasma glucose in zebrafish returned to resting levels 18h after simulated stress suggesting no long-term effects of this

stressor. These results are in agreement with Bracewell *et al.* (2004) who found that Chub (*Leuciscus cephalus*) species exposing simulated pulsed direct current electrofishing operations, plasma glucose levels were significantly higher at 0.5 hr after electrofishing operations, and showed a peak (11.0mmol/l) at 2 hr after treatment. They also found that glucose levels remained elevated up to 4 hr after treatments. In a similar study with the blood glucose responses on *Clarias gariepinus* exposing acute concentration of Glyphosate – Isopropylammonium (Sunsate), Okomoda and Atagoba (2011) showed that blood glucose concentration raised maximum 6.9±1.1 at highest concentration 20 mg/l. They also found a minimum glucose level in controlled experiment (2.9±1.2), which is similar to the present study. More the concentration of Glyphosate – Isopropylammonium in treatments results in more increases blood glucose concentration. The results also are in agreements with Barcellos *et al. (*1999) in the response to acute stress in Nile tilapia, *Oreochromis niloticus* (L.), previously exposed to chronic stress.

5.3 Red Blood Cell (RBC):

The physiological stress resulting from pH is clearly reflected by blood parameters of the experimental fish. Significant reduction in the values of red blood cell was observed in zebrafish exposed to different levels of pH. The reduction of the number of RBCs observed in this study affects the metabolism and normal function of the fish physiology. In a similar study on *Tilapia guineensis* Akinrotimi *et al.* (2010) also found reduced number of RBC (from $2.99\pm1.02\times10^6/\mu l$ to $1.78\pm0.21\times10^6/\mu l$) due to acclimation effect in that species which coincided with the findings of the present study. Akinrotimi *et al.* (2012) again found a decreasing number of RBC in *Tilapia guineensis* after exposing different salinity level. Similar observations have been reported by Bozorgnia *et al.* (2011) on common carp. They found that RBC decreased from the controlled one due to temperature dependence stress.

5.4 White Blood Cell (WBC):

Increased number of WBC was also found in the present experiment. WBC ranged from 4.39×10^3 cells/mm^3 to 7.84×10^3 cells/mm^3 in acidic treatments and 4.34×10^3 cells/mm^3 to 8.08×10^3 cells/mm^3 in basic treatments. Amount of WBC increased because they have to increase their immunity to adjust the stressful environment. Bozorgnia *et al.* (2011) showed an increased number of WBC due to acute effect of temperature on common carp. Similar increase of WBC was also recorded by Adeyemo (2007) in *Clarias gariepinus* due to lead effect. Akinrotimi *et al.* (2012) in *Tilapia guineensis* and Far *et al.* (2012) in Rainbow Trout (*Oncorhynchus mykiss*) also showed an increasing number of WBC at different stress.

5.5 Collection of eggs:

In the present experiment eggs were collected only from the acidic and controlled medium. Eggs collected from the acidic medium were possible when it was less acidic and the pH was near 6.0. This might be possible due to less toxicity in that medium. Eggs could not be collected from the basic medium which was highly toxic due to NaOH. Moreover pH in the basic aquarium was always near about 10.0 which might also be a cause of egg laying inhibition in basic medium.

5.6 Hatching rates:

In this experiment hatching of eggs was observed at pH 4-10. There was a wide range of pH in which hatching took place, suggesting that zebrafish eggs were more tolerant to extreme pH. Hatching rate was significantly higher ($P<0.05$) at pH 7.0 compared to those of pH 4.0, 5.5, 8.0 and 10.0. The hatching rates generally declined with decreasing acid concentrations and increasing alkalinity concentrations. Eggs became opaque and white within in a few minutes at pH 2.0 and 12.0. Gao *et al.* (2011) conforms a significant relationship to the present study. They conducted a similar experiment on pH effect on fertilization and hatching of Far Eastern Catfish (*Silurus asotus*) at pH ranging 2 to 13 under laboratory condition

and reported the highest hatching rates was at pH 7.0 and any increase or decrease of pH from 7.0 reduced the hatching rates. Carrick (1979) and Rask (1983) also conducted experiment with different fish species and found results similar to the present study.

5.7 Hatching time:

Zebrafish eggs can be hatched between 48 and 96 hr depending on the environmental temperature. In present study it was found that minimum hatching time (68 hr) was required at controlled pH. Highest hatching time (84 hr) was required at pH 4.0. Results related to hatching time are in agreement with Gao *et al.* (2011).They showed that minimum hatching time was required at controlled pH 7.0 in case of *Silurus asotus*. Similar findings were also reported by Rask (1983) in Perch (*Perca fluviatilis*) and Trojnar (1977) in brook trout (*Salvelinus fontinalis*).

5.8 Behavioral stress responses:

In the behavioral study it was observed that, the fish usually avoids the extreme high or low pH. They tried to aggregate at the corners of the experimental tank and also tried to find out any suitable place where they feel comfortable. Sometimes they jumped over the aquarium when they could not acclimatize with the environment and those remained in the aquarium became exhausted and died within 20-30 minutes. Similar findings were also reported by Serafy and Harrell (1993) after conducting field and laboratory experiment with three species, Banded killifish (*Fundulus diaphanus*), Blugill (*Lepomis macrochirus*) and Stripped bass (*Morone sexatilis*). They observed that in a gradual increase of pH, significant avoidance of the highest pH area was shown by all three species which was very much similar with the findings of the present study.

5.9 Skin color:

In the present experiment discolored skin of zebrafish was found in both acidic and basic medium. Skin of fish in both medium reared fish showed loss of normal appearance, brightness, gloominess and discoloration in their stripes. They showed a pale gloominess color in their skin. This might be due to the long term effects of acid or base in fish. Skin color effect was more pronounced in acidic medium than the basic medium.

CHAPTER VI

CONCLUSIONS

The main objective of the current study was to provide preliminary results on the sensitivity of zebrafish (*Danio rerio*) against pH stress effects. The findings of the present study demonstrated a direct correlation between pH exposures on some physiological changes in zebrafish. It is proved to be important to avoid possible misinterpretation of various pollutions to the physiological responses of fish. Such information can be used to evaluate the early effects and responses to acute chemical exposure reflecting the stressful conditions in the environment which can lead to fish health complication. Such tools appear to be an important method to approach and detect the effects of environmental toxicants on the response of the biological organization of fish.

The present study indicates that the fish were responding to the direct effects of the pH stress and secondary effects caused by stress. The findings of the present investigation demonstrated a direct correlation between pH exposure and some physiological changes in fish. And that the extents of these changes are proved to concentration and exposure time dependant. Such alterations like those observed in this study could result in severe physiological problems ultimately leading deterioration in health conditions and secondary infection and finally the death of fish.

The results obtained from this experiment can benefit the environmental monitoring of the aquatic ecosystems and aquaculture ponds by sampling fish to indicate any vulnerability in fish health. This can be established by developing a monitoring system of health status on fish before reaching critical point when exposed to waterborne pollution which can reflect their overall health status. Such a system can help to indicate any deterioration in the fish habitat which can inflect a decrease in health vulnerability resulting in an unbalanced internal homeostasis

conditions making them more susceptible secondary infections which can contribute to reduced growth and death.

Finally, the outcomes of this study will contribute largely to establish a monitoring program and a fish health laboratory at the Environmental Public Authority of Bangladesh to monitor the health conditions of the fish. A routine sampling program will help to monitor the wild fish species throughout the country to indicate any health complication prior to any fish mortality event by assisting the conditions and quality of fish. Such techniques would be more reliable than only using conventional methods by the use of water quality measuring equipment.

Since the strong water currents and large water body mass can help dilute the chemical pollutants rapidly altering their original concentration upon introduction. Therefore, the more reliable bioassay method could be developed that can help to detect such intoxication effects as an end point to the high environmental pH exposure areas even after the threat is removed. Also, some low concentration can be negligible having no effects on a short exposure period however at longer exposure periods can result in drastic biological effects and health complication. As a result, such bioassay monitoring program could help reveal and prepare for any environmental catastrophe.

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