Effect of acute physical and biological stressors on the haematological and biochemical responses in rohu, *Labeo rohita* (Hamilton, 1822)

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In
FISHERIES RESOURCE MANAGEMENT

By

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CERTIFICATE

This is to certify that the work embodied in the thesis entitled **"Effect of acute physical and biological stressors on the haematological and biochemical responses in rohu, *Labeo rohita* (Hamilton, 1822)"** *submitted by* **Miss. Chitra Pakhira** *in partial fulfillment of the requirements for the degree of* ***Master of Fishery Science (Fisheries Resource Management)*** *in the Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, is the faithful and bonafied research work carried out under my supervision and guidance. The results of the investigation reported in this thesis have not so far been submitted for any other degree or diploma. The assistance and help received during the course of investigation have been duly acknowledged.*

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APPROVAL SHEET

APPROVAL OF EXAMINERS FOR THE AWARD OF THE DEGREE OF MASTER OF FISHERY SCIENCE

(Fisheries Resource Management)

We, the undersigned, have been satisfied with the performance of Chitra Pakhira, in the Viva-Voce Examination, conducted today, the ..., 2013, recommended that the thesis be accepted for the award of the Degree of Master of Fishery Science in Fisheries Resource Management

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CONTENTS

List of Tables

List of Figures

List of Plates

CHAPTER-1

Introduction

1. INTRODUCTION

Fish evolved around 450 million years ago and currently they form the oldest and most diverse vertebrate group comprising approximately 32,500 recognized species globally. Fish is one of the most readily available and easily digestible protein sources available to man contributing nearly 20% of total animal protein consumed by world's population. In addition to protein, it is also an important source of carbohydrates, vitamins A and D, iron, calcium, and other mineral salts. The prominence of fish as food source has been mounting ubiquitously with increase in population and rapid expansion in food industry. It is often overlooked that more than 500 million people depend, directly or indirectly, on fisheries and aquaculture for their livelihoods. In addition, fish provides essential nutrition for about three billion people and is highly traded with more than 37% of production entering international trade (FAO, 2009).

India is bestowed with vast and varied inland fisheries resources comprising rivers and canals, reservoirs, ponds and tanks, floodplain lakes and wetlands and brackishwaters. Fisheries sector also plays a vital role in Indian economy through substantial foreign exchange earnings, employment generation (to about 14 million people) and ensuring nutritional and food security. The sector contributes to 1.1% of the GDP and 4.7% of the agricultural GDP. Fisheries sector in India has made rapid strides in recent years. Growing urbanization, globalization and rapidly changing social structures have had a major impact on the fisheries structure in the country. The total fish production has shown a phenomenal growth from a meager 0.75 million metric tonnes in 1950-51 to 8.67 million metric tonnes (Inland: 5.37 million metric tonnes and Marine: 3.30 million metric tonnes) in 2011-12 (Anon, 2013). India currently occupies 3rd position in fisheries and 2nd position in aquaculture production of the world next to China (FAO, 2013).

The world's capture fisheries and the livelihoods they support, however, are already under threat from a range of factors. Aquaculture is the world's fastest growing food production system, increasing at a rate of 8% annually. It has the greatest opportunity for increased growth in supply and production efficiency improvement compared to other agricultural production systems. Freshwater aquaculture in India is dominated by carps which contribute about 87% of the total freshwater fish production. The three Indian major carps namely catla (*Catla catla*), rohu *(Labeo rohita*) and mrigal (*Cirrhinus mrigala*) and three domesticated exotic carps such as silver carp (*Hypophthalmicthys molitrix*), grass carp (*Ctenopharyngodon idella*) and common carp (*Cyprinus carpio*) are dominated species of aquaculture. Rohu, *Labeo rohita* (Hamilton, 1822) is the most important among the three Indian major carp species used in carp polyculture

systems. It is widely cultured throughout India owing to its high commercial value, good growth rate, consumer preference, omnivorous feeding habit and acceptability to artificial diets.

Concomitant with the rapid growth, there has been an increased occurrence of problems that accompany all aquaculture endeavours, especially semi-intensive and intensive ones leading to diseases and economic losses (Schreck, 1996). Fish under aquaculture conditions are invariably subjected to physical, chemical and biological changes or stressors such as handling, crowding, transporting, change in water and sediment quality and microbial load (Mock and Peters, 1990). In aquaculture, handling fish is inevitable as it is associated with management procedures such as netting, weighing, grading, vaccination, disease treatments, etc. Similarly, transporting fish is multiphase and inevitable operation. The transportation of live fish is a widespread practice in aquaculture from hatchery to farms or from hatchery to markets. Optimising the packing density for a specified period of transportation is needed to avoid stress related mortality both during and / or after transportation. Bacteria (biological stressors) are part of microorganisms present in the ponds and their pathogenic potential may be altered under physical and chemical characteristics of environment (Walters and Plumb, 1980). *Aeromonas hydrophila*, a Gram negative and opportunistic bacterium may cause disease outbreak in several freshwater fish species including carps (Rahman *et al.,* 1997), particularly when the animal is under stress. It is the causative agent of the disease known as 'haemorrhagic septicemia or 'motile aeromonas septicemia' which is related to the lesions caused by bacterium including septicemia (Guz and Kozinska, 2004). Edwardsiellosis, a disease caused by *Edwardsiella tarda* infects fishes causing septicemia (Thune *et al.,* 1993; Yu, 2010). It has been isolated from number of farmed fish (Meyer and Bullock, 1973).

Exposure of fish to stressors can elicit physiological changes at multiple levels of animal organization, these alterations are collectively known as stress response. When fish are subjected acute or chronic stressors their immune system becomes weakened. Consequently their ability to fight disease is reduced and they then succumb to infections and fall sick. In severe or prolonged cases, this may lead to death. Acute stressors may lead to sudden impairment in fish physiology and in extreme cases fish succumb to death. Whereas, chronic cases are less obvious to the eye, but, result in reduced feeding response, higher feed conversion ratios and lower profit.

When fish are exposed to a stressor, the physiological stress response is initiated by the recognition of a real or perceived threat by the central nervous system (CNS). Then the sympathetic nerve fibers innervate the chromaffin cells, and stimulate the release of catecholamines via cholinergic receptors (Reid *et al.*, 1996). Because catecholamines,

predominantly, are stored in the chromaffin cells, their release is rapid and the circulating levels of these hormones increase immediately with stress (Mazeaud *et al.*, 1977; Randall and Perry, 1992; Reid *et al.*, 1998). The release of corticotropin-releasing hormone (CRH), or factor (CRF), chiefly from the hypothalamus in the brain, which stimulates the corticotrophic cells of the anterior pituitary to secrete adrenocorticotropic hormone (ACTH). Circulating ACTH, in turn, stimulates the interrenal cells in the kidney to synthesize and release corticosteroids or cortisol into circulation for distribution to target tissues. Then this cortisol influences the increase or decrease of the metabolic changes, osmoregulatory disturbance and changes in haematological parameters and ultimately impairment in immune function. All these physiological changes eventually lead to changes in whole animal performance characteristics such as growth, swimming capacity, disease resistance, feed intake and aggression behaviour.

Stress mitigation is one of the most challenging tasks in aquaculture and is the most promising areas of research. Knowledge on haematological and biochemical changes is very important in monitoring not only the stress and health status of fish, but also serves as diagnosis of metabolic disturbance and structural and functional status of the body (Blaxhall, 1972; Rehulka, 2002). Certain blood parameters serve as reliable indicators of fish health. To date, there have been several studies on the effects of stress on a variety of fish species in aquaculture (Barton *et al.,* 1980; Gbore *et al.,* 2006, Bailone *et al.,* 2010) However, detailed study on the effects of acute stressors on haematological and biochemical paramaters including metabolic enzymes of carps, especially rohu *Labeo rohita* is scanty (Chatterjee, 2012). Therefore, the present study is carried out with the following objectives.

1. To evaluate the haematological and biochemical responses of rohu, *Labeo rohita* subjected to acute handling.
2. To study the effect of transportation and packing density on the haematological and biochemical responses of rohu, *Labeo rohita*.
3. To study the haematological and biochemical responses of rohu, *Labeo rohita* experimentally challenged sublethally with bacterial pathogens such as *Aeromonas hydrophila* and *Edwardsiella tarda*.

CHAPTER-2

Review of Literature

2. REVIEW OF LITERATURE

2.1. Fish stress

Stress is an abnormal physiological condition of fish that results when the fish's collective adaptive responses to environmental factors are extended to, or approach its limit of tolerance. When fish are stressed, or continuously exposed to stress, their immune system becomes weakened. Consequently, their ability to fight disease is reduced and they then succumb to infections and fall sick. In severe or prolonged cases, this may lead to death. Stress can be acute or chronic. Chronic cases though less noticeable often result in reduced feeding response, higher feed conversion ratios, slower growth rate and, thus, lower returns in aquaculture systems.

In 1936 a scientist named Hans Selye, upon observing effects of noxious stimuli in laboratory animals, coined the term "stress" and defined it as *"the non-specific response of the body to any demand for change"* (Selye, 1936). Stress responses are most consistently observed in the gills, liver, blood, skin, and components of the urino-genital tract. In addition to presenting examples of various stressors and corresponding effects, this review highlights certain challenges of evaluating stress in fish.

2.1.1. Stressors

Throughout the animal kingdom, many types of stressors are universal simply because the basic needs of most animals are similar. Stressors may arise due to physical, chemical or biological factors. Universal stressors generally include deviations from optimal ranges for environmental parameters (e.g., ambient temperature, oxygen supply), insufficient food availability, inadequate refuge from sunlight or predators, and the demands of social interactions such as territorial disputes (Nasse and Young, 2000). Other stressors are unique to certain animal groups or habitats.

As compared to terrestrial inhabitants, fish and other aquatic creatures which live in a dynamic medium are subjected to a broader variety of stressors because their homeostatic mechanisms are highly dependent on prevailing conditions in their immediate surroundings. Stressors for fish may include fluctuations in water salinity, pH, hardness, alkalinity, dissolved solids, water level or current, and exposure to waterborne pathogens or toxicants. Fish reared in confinement systems often experience further pressures of crowding, handling, suboptimal nutrition and nitrogenous waste accumulation (Claudia *et al.*, 2009).

2.1.1.1. Fish-specific stressors

Fishes occupy a remarkably diverse array of habitats. Accordingly, environmental conditions that might be optimal for one species are inherently stressful for another. Given

the number of potential stressors, and the fact that fish may be exposed to multiple stressors simultaneously, the range of potential stress-inducing situations is almost limitless. This section provides brief descriptions of commonly encountered stressors and the anatomic sites in which corresponding morphologic effects tend to occur.

Procedures that can intensify the stress response in aquaculture fish include transportation, sorting, grading, vaccine administration and disease treatment if any (Burgess and Coss 1982). Additional stressful events include crowding, hypoxia, and physical trauma, after effects of anesthetics or sedatives and barometric disturbance in fish harvested at considerable depth. Evidence that these stimuli are intrinsically stressful is provided by experiments that have documented marked increases in blood cortisol and/or glucose levels in fish following deliberate handling and transport (Barton, 2002; Acerete *et al.*, 2004; Hosoya *et al.,* 2007). There may be some benefit to sedating fish before transport in order to mitigate shipping stress. In a study in which channel catfish (*Ictalurus punctatus*) were subjected to stressors such as confinement, high ammonia and oxygen depletion, sedation resulted in lower cortisol elevations than those observed in control fish (Small, 2004).

The blood of carp *Cyprinus carpio* (L.) showed lower haemoglobin and haematocrit values, higher erythrocyte sedimentation rate (ESR) in winter than spring (Murachi, 1959). Adaptation to elevated temperatures by fresh water teleosts brook trout, *Salvilinus fontinalis* (Mitchill) showed an increase in haemoglobin and erythrocytes level and a decline in total cell volume (Houston and Dewilde, 1969). Studying on the leucocytes numbers of cultured fish, Enomato (1969) found a decrease in lymphocyte numbers during oxygen deficient conditions, there was no difference in numbers before and after feeding, and numbers did not vary between heart blood and tail – cut blood. Dheer *et al.* (1986) observed that the higher levels of sodium chloride stress (3.2 g/l – 6.2 g/l) caused reduction in values of blood parameters.

The increase in ammonia level in blood and muscle tissue after exercise was reported in rainbow trout (Mommsen and Hockachka, 1988; Wright and Wood, 1988). No changes were observed in the number of erythrocytes, Hb or Ht in the fresh water *Labeo capensis* upon short exposure to sublethal (Hattingh, 1976) and lethal (Smart, 1978) levels of ammonia. Decrease in haemoglobin and haematocrit after acute exposure to ammonia has been reported for the African catfish *Clarias gariepinus* and blue tilapia *Oreochromis aureus* exposed to lead and copper (El – Nagar *et al.,* 2001) and *Ctenopharyngodon idella* exposed to acute toxicity of ammonia (Salah El – Deen, 1999).

Qayyum and Naseem (1967) reported lowest blood values (haematocrit, haemoglobin and erythrocyte number) in immature fishes. Vales increased as the fish advanced towards maturity, and the higher values were found in ripe fishes.

Siddiqui and Nassem (1979) studied the haematology of rohu, *Labeo rohita* and observed the haematocrit value ranging from 25.0 to 50.0% with a mean of 34.56%. The number of erythrocytes ranged from 1.65 to 2.93 (mean 2.29) million/$mm^{3,}$ males having more cells than females ($P > 0.05$). The leucocytes count ranged from 3,800 to 10,000 $mm^{3,}$ with a mean of 6,250/mm^3 and difference between male and female count were not significant.

2.1.2. Stress response

The sum of the physiological changes that occur in fish to physical, chemical or biological challenges and attempt to compensate is commonly referred to as the stress response (Wedemeyer *et al.*, 1990). The challenges themselves, if they are severe enough to require an energy-demanding compensatory physiological response by the affected fish, are referred to as *stress factors,* or *stressors*. Cumulative effect of stressors by acute or chronic that exceed acclimation tolerance limits of the fish will reduce the probability of survival, (Barton and Iwama, 1991).The physiological changes that occur as fish attempts for compensating stressors are similar in many ways to those occurring in the higher animals (Wedemeyer *et al.*, 1990). In response to a stressful event such as handling or crowding, the hypothalamic portion of the brain stimulates the pituitary to release adrenocorticotropic hormone (ACTH). ACTH is circulated to the anterior kidney where it stimulates the interrenal cells to produce cortisol and other corticosteroid hormones. The sympathetic nervous system stimulates the chrornaffin tissue of the anterior kidney to produce catecholamine hormones including 'adrenaline. Both classes of hormones initiate cardiovascular changes such as increased blood flow and pressure, increase oxygen consumption, and initiate secondary blood chemistry changes such as hyperglycemia. These physiological changes are adaptive in nature and normally enable the fish to compensate and improve its probability of survival. For example, the increased blood flow improves gill perfusion, to the extent that gas exchange is facilitated. A significant degree of immunosuppression also occurs as a side effect of the stress response and latent infections can become activated (Barton and Iwama, 1991).

Past research works indicate that certain stress responses are well conserved evolutionarily. In terms of behaviour, an obvious example is the instinctive urge to fight or flee when faced with an adverse stressor such as predation. Many physiological responses to stressors are also remarkably comparable among taxonomically diverse animals. For instance, common among all vertebrates is the stressor-induced secretion of adrenergic and glucocorticoid hormones; the latter especially is considered a hallmark of the stress response (Nasse and Young, 2000). Although fish lack adrenal glands per se, analogous production and release of adrenal cortical and medullar hormones occur in the inter-renal cells and chromaffin tissues, respectively, both of which are typically located in the piscine

anterior kidney. The activities of these hormones are clearly beneficial when acute action and its consequences take priority, as they elicit a heightened state of alertness, increase blood pressure and respiration, promote hepatic glycogen catabolism to provide a source of energy via glucose, and limit excessive tissue damage from inflammatory reactions to trauma or illness (Nasse and Young, 2000). However, hormonal stress responses that overcompensate or persist can also have negative effects, such as immune suppression, depletion of energy reserves, muscle breakdown, and, in fish, interference with osmoregulation as a result of altered mineral metabolism (Banerjee and Bhattacharya, 1995).

2.1.2.1. Stress response model

The biological model of stress response consists of three major components (i) recognition of stress to homeostasis (ii) the stress response and (iii) the consequence of stress. The longer the fish is stressed the longer the immune system is suppressed and greater the opportunity for disease outbreak. However, disease is only one possible pathological state. Other effects would be reduced feeding, inability to reproduce, abnormal behavior or the failure to grow normally. Fortunately most stressor last only a brief time and the biological cost of coping with them is relatively small (Moberg, 1985).

In order for fish to survive, their physiological systems must be capable of adjusting to challenges from the numerous naturally occurring changes in the chemical, physical, and biological conditions in the aquatic environment. These challenges can range from effects of though water chemistry alterations to behavioral conflicts (Wedemeyer, 1996).

A basic understanding of the physiology of the stress response, and the environmental alterations to which fish can adapt through this response, is important to identify stressful rearing conditions and develop methods to mitigate their adverse effects on health and physiological condition. In fish-transport operations, for example the corticosteroid hormone response that occurs has been used to show that the stress of the collection and loading procedures is more severe than the process of trucking itself (Maule *et al.*, 1958). Similar kinds of information can be used to identify and minimize the stressful effects of other aspects of intensive fish culture.

2.1.2.2. Paradigm of stress response

The general pattern of the stress response tends to be similar whether the challenge has resulted from fish cultural procedures (netting, handling, transportation, disease treatments), water chemistry changes (turbidity, pH, temperature), or behavioral factors (fright, dominance hierarchies). A convenient paradigm for the stress response (Figure 2) is likely to occur in three stages:

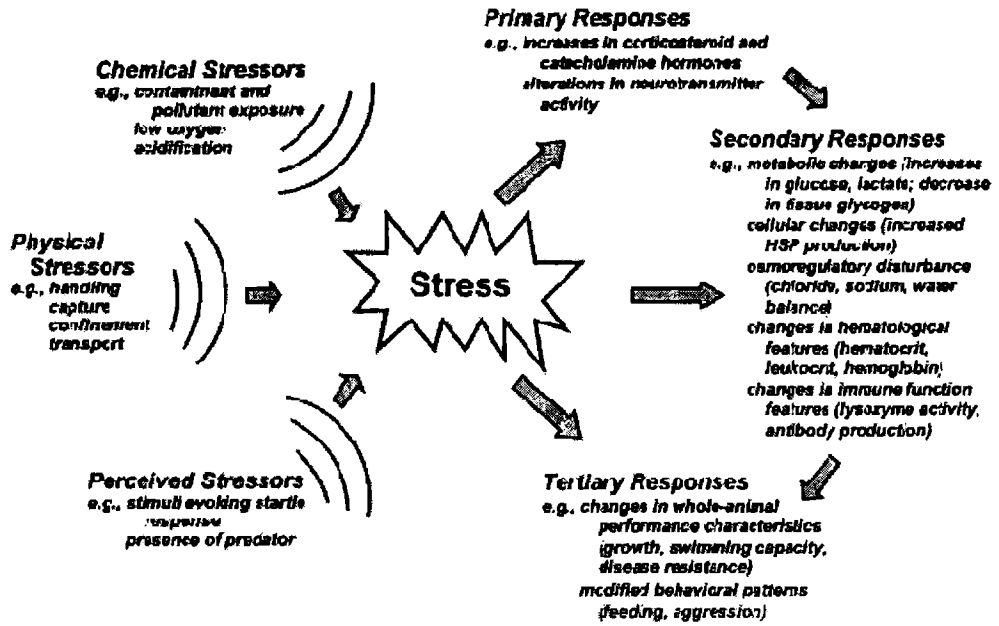


Figure 1. A typical paradigm for the stress response (Bruce *et al.*, 2002)

1. Primary Response or Initial Alarm Reaction (the endocrine system):

Following perception of a stressful stimulus by the central nervous system, corticotrophin releasing factor from the hypothalamus stimulates the pituitary to release adrenocorticotropic hormone (ACTH). ACTH is circulated to the interrenal cells in the anterior kidney and stimulates them to secrete cortisol. The chromaffin tissue of the anterior kidney is stimulated by the sympathetic nervous system to release adrenaline and other catecholamine hormones, which initiate a series of compensatory cardiovascular and blood chemistry changes.

2. Secondary Response or Stage of Resistance (Blood and tissue alterations):

Adaptive changes in blood and tissue chemistry and in haematology begin. These changes include increased gill Perfusion, elevated blood sugar (hyperglycemia) and reduced blood-clotting time. Maladaptive changes, such as lynaphopenia, hemorrhagic thymus and interrenal hypertrophy may eventually occur. Blood electrolyte losses due to diuresis may lead to life-threatening ion regulatory failure and circulatory system collapse. There is a caloric energy cost for compensation and growth may be reduced in case of prolonged stress.

3. Tertiary Response or Stage of Exhaustion (whole-animal changes):

The duration or severity of the stressful challenge has exceeded acclimation tolerance limits and the physiological changes needed to maintain homeostasis have become maladaptive. Immune protection is impaired and eventually may results in reduced growth, impaired resistance to infectious diseases. Behavioral changes such as impaired reproductive behavior may also lead to reduced survival.

4. Quaternary Response (populations, ecosystem):

Recruitment to succeeding life stages may be decreased sufficiently to result in population declines. At the community or ecosystem level, 'disruptions in energy flow through trophic levels may eventually result in altered species composition.

2.1.3. Measurement of Stress Responses

Various methods have been adopted for evaluating stress responses in fish. Some of them have been enlisted below

- Behaviour and whole body or organ weight measurements like condition factor, hepatosomatic index, and gonad somatic index (Dutta *et al.,* 2005; Hosoya *et al.,* 2006; Spencer *et al.,* 2008).
- Biochemical assays or hematological assays such as plasma cortisol, corticosterone, glucose, tissue damage enzymes, heat shock proteins, Lactate

dehydrogenase (LDH), Aspertate amino transferase (AST) and Alanine amino transferase (ALT) (Acerete *et al.,* 2004; Barton, 2002; Dutta *et al.,* 2005; Hosoya *et al.*, 2007; Iwama *et al.,* 2004; Olse *et al.,* 2008; Trenzado *et al.,* 2008).

- Immune function (Choi *et al.,* 2007).
- Gene expression patterns (Basu *et al.,* 2001, 2002; Van der Meer *et al.,* 2005);
- Measurement of fish steroids in water (Scott and Ellis, 2007); and
- Histology (Dutta *et al.,* 2005; Hosoya *et al.,* 2007; Spencer *et al.,* 2008).

2.1.3.1. Haematological and biochemical indices as stress markers

The individual physiological changes occurring during the stress response can be used to identify rearing conditions that promote the health and physiological condition of fish in intensive culture as well as those with potential adverse effects (Table 1) (Wedemeyer *et al.,* 1990).

The secondary blood and tissue chemistry changes that occur as the result of endocrine activity can also be used to characterize the severity of stress and the time fish need for recovery. There are two main advantages: The analytical methods are usually simpler and less expensive, and secondary changes provide physiological information integrated at a higher level of biological organization: The most commonly measured secondary changes are serum enzymes to detect organ and tissue damage, blood glucose as an indirect measure of stress hormone activity, and blood electrolytes such as Cl^- to, evaluate effects on ion regulation. Glucose and chloride determinations can also be useful in guiding the development of stress-mitigation procedures for fish cultural procedures, such as the use of mineral salt -foundations when transporting fish. The accumulation of lactic acid in muscle or blood (hyperlacticemia) is also well accepted as an indicator. Lactic acidosis occurs when excessive swimming activity has been severe enough to have caused an oxygen debt (Dror *et al.,* 2006; Ellaser and Clem, 1987).

Hematological determinations can also provide useful information about the tolerance of fish to an applied stress factor. For example, acclimation to temperature-induced increases in oxygen demand by salmonids is commonly accompanied by erythropoesis and increases in blood haemoglobin. However, the changes are relatively modest by comparison with cardiovascular and ventilator responses and probably make a minor contribution to acclimation (Houston *et al,* 1969). Changes in the blood erythrocyte, count (as approximated by the haematocrit), or in hemoglobin values following acute stress can also indicate that haemo-dilution or haemo-concentration has occurred (Fast *et al.*, 2006). Of all the haematological measurements, blood-clotting time and changes in the differential leucocytes count are among the most 'sensitive indicators of write stress. Increased leucocytes counts (leucocytosis) are not

Table 1. Physiological test and interprefive guidelines to assess effect of environmental factors on fish health and physiological status (Wedemeyer *et* al,. 1990).

Physiological test	Diagnostic significance if results are	
	Low	High
Erythrocytes	Anemia, haemoglobin, impaired osmoregulation	Stress polycythemia
Leukocytes	Leucopenia due to acute stress	Leukocytosis due to bacterial infection
Haemoglobin	Anemia, haemodilution	Haemoconcentration, gill damage
Haematocrit	Anemia,	Haemoconcentration, gill damage
Glucose	Inanition	Acute or chronic stress
Cholesterol (plasma)	Gill chloride cell damage compromised, osmoregulation	Haemoconcentration comprised osmoregulation
Cortisol	No recognized significance	Acute or chronic stress
Total protein	Infection, nutritional problems, renal failure	Haemoconcentration

normally associated with stress, but leucopenia does occur during the compensatory response. In general, stress results in lymphoperia, monocytoperia, and neutrophilia. The end results of all these effects are suppression of the immune response and increased susceptibility to diseases (Weclerneyer and Goodyear, 1984).

Changes in metabolic rate as an index of performance capacity affected by stress can also be estimated by measuring oxygen consumption rate usually in a respirometer. This provides a basis for estimating the effects of stress on bioenergetics budget and thus on scope for growth and activity. Nitrogen excretion, amount of food ingested and the gut absorption efficiency are measured and converted to equivalent units of energy (joules per hour) (Gbore *et al.,* 2006; Greenwell *et al.,* 2003; Groff and Zinki, 1999; Scott and Ellish, 2007; Cossins *et al.,* 2009).

2.1.3.1.1. Haematological parameters

Fish live in very close contact with the environment, and, therefore, they are susceptible to physical and chemical changes which may be reflected in their blood components (Wilson *et al.,* 1998). A thin epithelial membrane separates from the water and any unfavourable change in the water body is reflected in the blood. The amount of fish blood generally ranges to 5% of its body weight.

The use of haematological parameters as fish health indicator has been proposed by Hesser (1960) and blood tissue of fish gives clue about physiology and environmental condition of fish (Ramawamy and Reddy, 1978). Variations in blood tissue in fish are caused by environmental stress (Hille, 1982; Aldrin *et al.*, 1982) malnutrition (Casillas and Smith, 1977), gender (Siddiquie and Nasim, 1979; Collazos *et al.*, 1998), fish size (Garcia, 1992), seasonal difference and breeding (Cech and Wohlschlog, 1981). In order to determine haematological value of fish, some characteristics of fish and the environment should be considered. Haematological studies help in understanding the relationship of blood characteristics to the habitat and adaptability of fish species to the environment. The red blood cell count, haematocrit and haemoglobin concentration vary with diet, temperature, season and nutritional status of fish (Barnhart, 1969).

Many fish develop compensatory physiological adjustment for maintenance of respiratory homeostasis upon exposure to abnormal water quality parameters, which leads to increase in plasma glucose concentration and a reduction in the blood protein and haemoglobin concentration (Wood *et al.*, 1989; Wilkie and Wood, 1991; Wilkie *et al.*, 1993) followed by their recovery.

2.1.3.1.1.1. Total Erythrocyte Counts (TEC) or Red Blood Corpuscles (RBC)

The main function of red blood borpuscles is to serve transport energy to the cells in terms of oxygen and removing carbon dioxide from the body. The RBC makes up the majority of the cells. Fish RBC are nucleated, which comforts a life span of about 1.5 yrs (Bowser, 1993).

Red blood corpuscles counts were once a routine investigation in human medicine, but because of their inherent errors these have been widely replaced by the estimation of haemoglobin and haematocrit values and electronic methods have taken over from visual counts. However, with fish blood, owing to presence of both nucleated erythrocyte and leukocytes, electronic methods of differential counting are not feasible. The most commonly used method is the visual counting of cells in a neubaur haemocytometer. Difference dose arise due to inability to lyse selectively of the erythrocytes and leukocytes (Hesser, 1960; Mulcahy, 1970).

It has been reported, that Asphyxia leads rapidly to an increase in erythrocyte, haemoglobin and other blood constituents. Some degree of inverse correlation between erythrocytes cell size and counts in freshwater teleosts has been recorded (Smith *et al.*, 1987). The pike *Esox luciles* seems to have a greater number of cells than most other species with mean value of 18,93,000/mm^3 (Mulcahy, 1970). Sex differences have also been noticed in pike. However, the means for two sexes were differed narrowly. The values for erythrocytes in fishes are given as $5 \times 10^5 - 26 \times 10^5/mm^3$ with considerable degree of variation among the species. Correlation between metabolic activity and erythrocyte count has been well documented in fish (Eisler, 1965).

The amount of erythrocyte count in blood also varies in accordance to life habit. The fast swimming species of fishes on an average have more erythrocytes, larger haematocrit and more haemoglobin compared to less mobile forms; low oxygen in the environment stimulates erythropoesis and, thus, values for the Haematocrit and haemoglobin increase in freshwater fishes, which are transferred to water with low oxygen concentration. Henry *et al.* (1964) by isotope labelling, estimated a life span of erythrocyte is more than 150 days in experiment on tench (*Tinca tinca*).

Sopinska (1984) found that the normal value of RBC count in carp ranges between 1.32×10^6/ml-1.4×10^6/ml of blood in normal condition which could increase up to 1.63×10^6/ml of blood when fish is susceptible to various types of handling stress. Das and Mukherjee (2008) reported a decrease in RBC count from 2.09×10^6/ml to 1.94×10^6/ml in rohu (*Labeo rohita*) subjected to chronic stress (exposed to nuvan). In case of common carp the erythrocyte number normally ranges between 1.12×10^6/ml-1.17×10^6/ml (Dobsikova *et al.*, 2009). The RBC number decreased from 1.4×10^6/ml to 1.38×10^6/ml when fish are subjected

to long distance (12 hrs) transportation stress (Dobsikova *et al.,* 2006). Paurgholam *et al.* (2013) found that when grass cap (*Ctenopharyngodon idella*) is vaccinated with *Aeromonas hydrophila* following exposure to sub lethal concentration of Diazinon, for 30 days the RBC count decrease from $1.5x10^6$/ml to 1.4×10^6/ml. The RBC count in *Pangasionodon hypopthalamus* decreased from 4.66×10^6/ml to 4.21×10^6/ml when they were subjected to A*eromonas hydrophila* infection for 30 days (Phanikumar *et al.*, 2013), where as in *Oreochromis niloticus* it increased from 1.02×10^6/ml to 1.7×10^6/ml (Bailone *et al.*, 2010).

Banergee (1966), found the erythrocyte count to be higher in males than in females of *Anabas testudineus* and the same trend was found by Qayyum and Naseem (1967) in *Cirrhinus mrigala*. But in contrast Bagchi and Ibrahim (1974) reported that the erythrocyte to leukocyte ratio was found to be approximately 310:1 in female *Labeo rohita*. Antarctic fishes, the blood contains 0.38-1.2$\times 10^6$ erythrocyte is less than the normal values for teleosts (Huraea, 1966; Barber *et al.,* 1981). Average number of red blood cells in a number of marine teleosts of Puerto Rico was 2.3-5.3$\times 10^6/\mu l$ (Saunders, 1983). Elasmobranches in general show lower erythrocyte number in the blood than teleosts. (0.1-0.4$\times 10^6/\mathrm{ml}$ (Grodzinski and Hoyer, 1983). However, in the north Atlantic region chondricthyans (shark and rays) and holocephalans (chimaera) show not only lower haematocrit but also haemoglobin values than the teleosts (Fange, 1978).

2.1.3.1.1.2. Total Leucocyte Counts (TEC) or White Blood Corpuscles (WBC)

The white blood corpuscles perform the most vital role by preventing from any external stress as it associated with cellular immunity and with the production of humoral antibody (Wedemeyer *et al.*, 1990).

The leukocyte counts of fish blood is considerably higher than that of a man or other vertebrates (Andrews, 1965), accounting to more than $10^5/mm^3$ in some bony fishes (Puchkov, 1964). Puchkov (1964) also stated that the numbers in the same species of fish will vary greatly with age, season and sex gland maturation. The pike, *Esox lucius* (L.) appears to have a high count ranging from 79,000/ mm^3 – 1,37,000/mm^3 with the male showing a wider range than the female. Leucocytes suffer from a fair degree of inherent error when carried out visually and so great reliance is placed on haematocrit and haemoglobin estimation as indicators of anaemia. The leukocyte were in the range of 118$\times 10^4/mm^3$ for brown trout *Salmo trutta (L.)* , 137$\times 10^4/mm^3$ for rainbow trout *Salmo gairdneri* (R.) and 126$\times 10^4/\mathrm{mm}^3$ for brook trout, *Salvelinus fontinalis* (Mitchill) (Benfey and Biron, 2000) .

Leukocyte levels could be used as an indicator of some disease or pollution process upon the fish. Works concerning differential cell counts seems to have been carried

out on gold fish *Carassius auratus* (L.) (Watson *et al.*, 1963). Weinerb and Weinerb (1969) used leukocyte count changes as a means of assessing the systemic response of rainbow trout *Salmo gairdneri* (R) to various injections.

High white blood cell counts indicate damage due to injection of body tissue, serve physical stress, and as well as leukemia. Mishra and Srivastava (1980) reported an increase in leukocyte count in rainbow trout when they exposed to heavy metals. Some of the most common causes of heavy metal toxicity are inflammatory lesions associated with tissue damage, anemia and neoplasia.

According to Phanikumar *et al.* (2013), WBC count in *Pangasionodon hypopthalamus* increased from 3.91×10^3/ml to 6.058×10^3/ml when they were subjected to *Aeromonas hydrophila* infection for more than 30 days, where as in *Oreochromis niloticus* it increased from 1.7×10^3/ml to 3.7×10^3/ml due to *Aeromonas* infestation (Bailone *et al.*, 2010). Das and Mukherjee (2008) reported a increase in WBC count from 19.77×10^3/ml to 27.79×10^3/ml in rohu (*Labeo rohita*) subjected to chronic stress (exposed to nuvan).

According to Jin Ha Yu (2010) When *Silurus asotus* were subjected to infestation of *Edwardsiella tarda* the WBC count increased from 18.8×10^3/ml to 33.1×10^3/ml. Paurgholam *et al* (2013) found that when grass cap (*Ctenopharyngodon idella*) is vaccinated with *Aeromonas hydrophila* following exposure to sub lethal concentration of diazinon, for 30 days the WBC count decrease from 26.7×10^3/ml to 18.58×10^3/ml. The WBC number increased from 57.56×10^3/ml to 79.94×10^3/ml when common carp (*Cyprinus carpio*) are subjected to long distance (12 hrs) transportation stress (Dobsikova *et al.*, 2006). In case of common carp (*Cyprinus carpio*) the leukocyte number normally ranged between 50.5×10^3/ml to 57.8×10^3/ml (Dobsikova *et al.*, 2009). Sopinska (1984) found that the normal value of WBC count in carp ranges between 33.75×10^3/ml of blood in normal condition and 45.01×10^3/ml of blood when fish is susceptible to various types of handling stress. Puchkov (1974) stated that the numbers in the same species of fish will vary greatly with age, season and sex gland maturation. He reported that pike *Esox lucius* (L.) appears to have a high count among other fishes, ranging from 79000/ mm^3 – 137000/mm^3 with the male showing wider than the female (Mulcahy, 1970).

2.1.3.1.1.3. Haematocrit

Haematocrit (Ht) is a measure of cellular function of blood and is determined as the packed cell volume. The increase in heart beat and the need for higher oxygen intake could cause an increase in number of moving erythrocytes and, thereby Haematocrit value.

This is also used as stress index because it is very simple to determine even if standard values have to be validated for each species before they can be correctly used (Reddy and Letherland, 1995).

Increase in Ht values may indicate stress induced spleenic release of red blood cells. The Haematocrit value of fish blood generally ranges from almost 0 to more than 50% in actively swimming, and surface feeding fishes. However in most teleosts it ranges between 20% and 40%. The haematocrit, hemoglobin concentration and other hematological parameters are highly sensitive to physiological changes, for instance those occurring due to stress (Soivio and Oikari, 1976). Contraction of spleenic vessels may force stored red cells into the general circulation (Fange and Nilson, 1985) in stress conditions.

Sopinska (1984) found that the normal value of haematocrit value in carp ranges between 27.13% and 35.80% which could be as high as 44.50% and 49.80% when fish is subjected to various types of handling stress. Paurgholam *et al.* (2013) found that when grass cap (*Ctenophayngodon idella*) is vaccinated with *Aeromonas hydrophila* following exposure to sub lethal concentration of diazinon, for 30 days the PCV value decrease from 22.6% to 19.7%. According to Jin Ha Yu (2010) when *Silurus asotus* are subjected to infestation of *Edwardsiella tarda* the PCV value decreased from 31.6% to 14.7%. When *Oreochromis* was subjected to *Aeromonas* infestation the PCV value increased from 18.83% to 21.86% (Bailone *et al.*, 2010). According to Phanikumar *et al.* (2013), PCV value in *Pangasionodon hypopthalamus* decreased from 42.79% to 35.16% when they are subjected to A*eromonas hydrophila* infection for more than 30 days.

2.1.3.1.1.4. Haemoglobin

Fish hemoglobin is a possible indicator of oxygen -binding capacity of the blood. Though it is easy to measure but it is not a very sensitive stress indicator and as a result of this mostly haematocrit value is preferred for estimation of stress (Bruce *et al.*, 2002). Dobsikova *et al.* (2006) found Hb content 9.4 g% in case of normal common carp (*Cyprinus carpio*).

Apart from the special case of the ice fishes, most oxygen in the blood is carried by blood hemoglobin which results from p^H dependent configuration changes in the haemoglobin molecules, and oxygen is unloaded at the site just where it is needed by the fish (Chpman and Hall, 1996).

Hagfishes and lampreys have monomeric haemoglobin, but in all other fishes it is tetrameric (as they are in the mammals). Different hemoglobin may occur in one fish, perhaps to adopt the gas transport system to changing conditions, as in the migratory American eel (*Anguilla rostrata*) where haemoglobin molecule has a high oxygen affinity in seawater than in freshwater (Chapman and Hall, 1996).

The anaemic condition in fish results from an un-usually low number of red blood cells or low concentration of haemoglobin in the red blood cells. Significance of the changes

may be understood in terms of reduced oxygen consumption in fish resulting in death due to heavy metal pollution (Christensen *et al.*, 1972).

Das and Mukherjee (2008) reported a decrease in haemoglobin from 5.77 g/dl to 3.47 g/dl in rohu (*Labeo rohita*) subjected to chronic stress (exposed to nuvan). According to Jin Ha Yu (2010) when *Silurus asotus* are subjected to infestation of *Edwardsiella tarda* the Hb value decreased from 3.25 g% to 60 g%. According to Phanikumar *et al.* (2013), Hb value in *Pangasionodon hypothalamus* decreased from 14.03 g% to 12.11g% when they are subjected to A*eromonas hydrophila* infection for more than 30 days. Paurgholam *et al.* (2013) found that when grass cap (*Ctenopharyngodon idella*) is vaccinated with *Aeromonas hydrophila* following exposure to sub lethal concentration of diazinon, for 30 days the Hb value increased from 5.0g% to 6.5g%.

2.1.3.1.2. Biochemical indices as stress markers

2.1.3.1.2.1. Glucose

Glucose is a carbohydrate that has a major role in the bio-energetic of animal being transformed to chemical energy (ATP) which in turn can be expressed as a mechanical energy (Luycas, 1996). Blood glucose has been shown to be a sensitive biochemical indication of environmental stress for a number of pollutants (Table 2). The blood sugar level represents a dynamic balance between the rate at which the sugar is entering into the blood stream from the liver and the rate at which it is being removed by the body tissue from the blood.

Bakhtavathsalam and Reddy (1982) reported that the increased blood sugar level contribute an active flux of metabolites. Greaney (1978) has also observed that different concentrations of toxicant interfere carbohydrate metabolism. The elevated blood glucose levels reflect an increase in the rate of muscle carbohydrate where high energy demand was met due to brisk and erratic movements (Ravichandran *et al.*, 1995). When fish absorbs little oxygen from the environment, the respiratory metabolism is depressed and therefore, stored intracellular glycogen is utilised. Under such condition, the hyperglycaemic hormone is released for the degradation of glucose. This glucose leaks into the blood causing hyperglycaemia (Bhattacharaya *et al.*, 1987).

Glucose increase is a general response of fish to acute pollutants, including organophosphates (Svobodova, 1999; Srivastava, 1981; Singh and Srivastava, 1982; Mishra and Srivastava, 1980; Nataranjan, 1989; Gill *et al.*, 1990; Babient *et al.*, 1995; Sancho *et al.*, 2000). Plasma glucose level increased during the acute (96 hrs) but not affected during the sub chronic (8 weeks) exposure of selenium (Hossam *et al.*, 2009). The stress hormones

Table 2. Plasma glucose (mmol/l) values of different species before and after stress

Species	Stressor	Pre-stress	Post-stress	Exposure	References
Bald notothen (*Pagothenia borchgrevinki*)	Temperature	0.5	10	Acute	Lowe and Davison (2005)
Coral trout (*Plectropomus maculates*)	Capture and handling	0.6	0.9	Chronic	Frisch and Anderson (2005)
Emerald rockcod *Trematomus bernacchii*	Temperature	0.5	0.5	Chronic	Lowe and Davison (2005)
Rainbow trout (*Oncorhynchus mykiss*)	Copper and air exposure	0.1	0.2	Chronic /Acute	Gagnon *et al.* (2006)
Nile tilapia (*Oreochromis niloticus*)	Electroshock	0.2	0.4	Acute	Barreto and Volpato (2006)
Nile tilapia (*Oreochromis niloticus*)	Social stressor	0.9	0.7	Acute	Barreto and Volpato (2006)
Atlantic cod (*Gadus morhua*)	Nitrite exposure	0.17	0.23	Chronic	Siikavuopio and Sæther (2006)
Channel catfish (*Ictalurus punctatus*)	Handling	0.7	0.8	Chronic	Welker *et al.* (2007)
White sturgeon (*Acipenser transmontanus*)	Air exposure	0.6	0.7	Acute	Zuccarelli *et al.* (2008)
Common carp (*Cyprinus carpio*)	Transportation	0.2	0.7	Acute	Dobsikova *et al.* (2009)
Rohu (*Labeo rohita*)	Transportation	0.2	0.8	Acute	Chatterjee *et al.* (2010)

such as catecholamines, cortisol and other may be influenced by the internal or external conditions in the history of the fish (anoxia, pollution, nutritive stress, physical stress) (Reid *et al.,* 1998) and known to increase blood glucose, (Van Raaji *et al.,* 1995).

Though the glucose level is an indicative to acute and chronic stress response to the teleosts, however, it is less responsive to elasmobranches. Amount of glucose level may be influenced by species, rearing history and other environmental factors such as temperature and diet (Chatterjee *et al.,* 2009).

Barnhart (1969) suggested an average blood sugar normal range of 75 – 150 mg/100ml for Rainbow trout. Svoboda (1999) showed that the glucose level increased in common carp from the level of 5.5 mg/dl to 7.9 mg/dl when they were subjected to handling and transportation stress. Datta *et al.* (2002) observed that plasma glucose level increased as a result of glycogenolysis in muscle and liver of *Labeo rohita.* When *Oreochromis* subjected to *Aeromonas* infestation the glucose value decreased from 85.42mg/dl to 42.81mg/dl. According to Rehulka *et al.* (2002) the normal plasma glucose level for rainbow trout is 4.63 mmol/l. According to Jin Ha Yu (2010) when *Silurus asotus* are subjected to infestation of *Edwardsiella tarda* the glucose value decreased from 35.5 mg/dl to 14mg/dl.

2.1.3.1.2.2. Total proteins

The serum proteins in fish blood have not been studied in details in fishes. Among the serum proteins identified, most of the fish serum contain albumin like protein ,a number of ca^{2+} binding proteins (like Ceruloplasmin, Vitllogennin), blood clotting proteins (like fibrinogen and prothrombin), metal binding protein (like transferrin), immunoglobulin, lipoproteins (High density lipoproteins HDL, and Low density lipoproteins LDL) and hormone binding proteins (steroid binding proteins) (Chapman and Hall. 1996).

Apart from albumin or para-albumin, there is very little information available on phylogenic distribution of the proteins. Most of the information about these proteins is descriptive, (i.e. molecular weights, binding characteristics, electrophoretic mobility), with very little information available as to the amount in plasma.

Serum protein forms approximately 80% of the blood solutes. The plasma protein in fish ranges from 2-8 g/dl (Flecher, 1984; Farrell *et al.,* 1983; Miller *et al.,* 1963; Sandnes *et al.,* 1988; Hunn and Greer, 1990) and appears to be fairly constant within and among the fish species. The physiological range of total protein in case of *Cyprinids* normally ranges between 2.10 g/dl to 5.76 g/dl (Nicula *et al.,* 2010).

The plasma protein is altered mainly by changes in plasma volume. An increase is caused by a shift of fluid from the plasma to the intracellular compartment, and a decrease may be due to hydration in plasma .The fluid shifts out of the plasma is caused by an

osmotic imbalance between the extracellular and intracellular compartments and any stress that induce such an imbalance can lead to increase in plasma protein. Total serum protein increased in rainbow trout in response to strenuous exercise and exposure to low pH (Milligen and Wood, 1982). Reduction in plasma protein is generally associated with prolonged starvation (Love, 1982) and severe stress (Stevens, 1968). Increased total protein is generally associated with dehydration, and in some cases of chronic liver disease. Decreased protein level might associate with starvation and mal absorption or mal nutrition (Pagana and Kathleen, 1998). According to Rehulka (2002) the total plasma protein level for rainbow trout is 10.8 g/dl.

2.1.3.1.2.3. Cholesterol

Most of the fishes with the exception of elasmobranchs and garpike, are hypercholesterolemia. In the agnathids, approximately 50% of the cholesterol is carried by LDL (Low density lipoprotein), whereas in elasmobranchs and teleosts, 60-90% of the cholesterol is constituted by HDL (High density lipoprotein) (Babin and Vernier, 1989).

Plasma cholesterol levels show considerable intra-specific as well as inter specific variability. For example serum cholesterol in Pacific salmon varied from 300 mg/dl to 1470 mg/dl (Robertson *et al.*, 1961) and in the Atlantic cod values ranged from 399 mg/dl to 1598 mg/dl (Larsson and Fange, 1977). These extreme individual variations are no doubt linked to difference in diet, activity, and sexual development. Generally, the highest plasma cholesterol levels tend to be seen in both male and female prespawing fish and also appear to be linked to the active feeding. Upon spawning, plasma cholesterol tends to drop, though more so in females than in male. In female migrating Pacific salmon, plasma cholesterol reached highest value of 635 mg/dl, but in spawned fish, cholesterol levels dropped precipitously, to 126 mg/dl (Robertson *et al.*, 1961).

Although not as extensively studied, nonsalmonid teleosts tend to show the same general trends: In prespawing sea bass, plasma cholesterol levels peak at about 900 mg/dl and fall to about 350 mg/dl in spawning fish (Fernandez *et al.*, 1989). Similarly, in plaice (White *et al.*, 1986) and the stripped mullet, *Mugil cephalus* (Digiulio, 1993), peak plasma cholesterol levels (225 mg/dl and 300 mg/dl,) were seen in the summer month when fish were actively feeding and lowest levels (150 mg/dl) were seen when fish were spawning. According to Rehulka *et al.* (2002) the plasma cholesterol level for rainbow trout is 0.72mmol/l.

2.1.3.1.2.4. Triglycerides

Triglycerides (TG) are the primary storage of lipid in most fish species and are readily mobilized in response to physiological demand. Triglycerides released from storage site are transported in the plasma in association with LDL (Low density lioprotein). Fatty acids released from triglycerides by the action of extra cellular lipases, are taken up by the tissue, and the glycerol is transported back to the liver via the plasma.

As is case with the other lipids, the level of plasma TG varies throughout the life cycle of fish and is affected by such factors as sexual maturation, smoltification (in the salmonids), spawning and nutritional status. In the salmonids, smoltification generally results in a depletion of body lipids, reflected in a reduction in plasma TG levels from 1100 mg/dl in parr to 700 mg/dl in smolts (Sheridan, 1989).

In all teleosts species examined, the highest levels of plasma TG are associated with the period of peak feeding in preparation for spawning. In feeding prespawing plaice, channel catfish, and Arctic char, plasma TG levels peaked at 100 mg/dl, 600 mg/dl and 750 mg/dl respectively (Mckay *et al.*, 1985; White *et al.*, 1986). During the period of spawning, plasma TG levels generally decline by about 20% to 50%. The extent of the decline appears to related to the levels of activity during spawning. The more active fish (e.g. Arctic char) experience a greater decline in TG levels (McKay *et al.*, 1985; Sheridan, 1989).

Longer the starvation period, greater is the decline in triglecride levels. In sea bass starvation for 40 days, plasma TG levels dropped from 560 mg/dl to 280 mg/dl and after 150 days, TG levels fell to about 70 mg/dl (Zarmmit and Newsholme, 1979). Likewisein Arctic char that have ceased feeding due to low temperature, plasma TG levels fell by about 60% to 200 mg/dl after 60 days (Danulat *et al., 1989*).

Generally elasmobranchs tend to have lower plasma TG levels than do teleosts and are less depend on physiological and nutritional state of the animal. In spotted and spiny dog fish, plasma TG levels ranging from 30 mg/dl to 120 mg/dl (Zammit and Newsholme, 1979; Garcia *et al.*, 1992) and at least in the spotted dogfish, were unaffected by 100-150 days of starvation (Zammit and Newsholme, 1979) or sexual maturation (Garcia *et al.,* 1992). The difference may be explained by the fact that elasmobranches do not mobilize lipid reserve as triglycerides, but rather as ketone bodies, which is supported by the absence of detected free glycerol in the plasma (Zammit and Newsholme, 1979). According to Rehulka (2002) the plasma triglycerides level for rainbow trout is 0.47mmol/l.

2.1.3.1.2.5. Urea

The sources of urea which serves as the animal's most abundant organic osmolyte is synthesised by the ornithine urea cycle in the liver. In some euryhaline elasmobranchs plasma urea level reduces when animals are placed in dilute seawater and conversely and increases when fish are exposed to concentrated seawater. In lemon shark, plasma urea, however, remained constant in the face of changing salinities (Watts and Watts, 1974; Perlman and Goldstein, 1988).

A reduction of food intake over a period of several weeks can reduce plasma urea in elasmobranchs and hence their ability to hyperosmoregulate. Plasma urea increased almost immediately after refeeding (Hay Wood, 1973).

In freshwater fishes urea is present in very low levels (1 – 10 mM) and plays a significant role in osmoregulation. Uric acid is formed by the degradation of purines, primarily in the liver and white muscle. Uric acid is generally converted to urea for execration so serum level of urea becomes typically low in the amniotelic fishes like carps. In rainbow trout, plasma uric acid ranges from 40-100mM (Hille, 1982). Plasma uric acid is not commonly measured therefore data available on phylogenetic trends, effects of starvation, and seasonal variation are very mearge.

According to Rehulka (2002) the plasma urea level for rainbow trout was found to be 0.9 mmol/l under control condition. In *Cyprinus carpio* urea level ranged between 0.97 mg/dl and 1.43 mg/dl under normal condition but increased up to 2 mg/dl to 2.64 mg/dl under different environmental stressed condition, (Yang *et al.,* 2003). The urea nitrogen level could increase 1.2 mmol/L to 1.7 mmol/L when subjected to nitrite poisoning (Kubuley *et al.,* 2002). According to Jastrzebska *et al.* (2011) urea level in common carp might vary between 1.66 mg/dl and 6.33 mg/dl.

2.1.3.1.2.6. Creatine and creatinine

Creatine is an amino acid that is an end product of the metabolism of glycine, arginine and methionine and is found primarily in the white muscle. It's levels in plasma are typically low, 10 mmol/l 80 mmol/l. (Sandnes *et al.,* 1988), and appear to be unaffected by stress (Wells *et al.,* 1977). Creatine is not metabolized further and is execrated by kidneys (Daly *et al. 1980 &1985*). Creatinine is a precursor for the high energy phosphate or phospocreatine. The source of creatine in fish is unclear.

The necessary enzyme for synthesis of creatine have been found in carp, but are absent or undetectable in the pacific hag fish (*Eptatertus*), a shark (*Prionus sp*) and ray (*Urolophus sp*) (Van pilsum *et al.,* 1972). Creatinine is known to be supplied in sufficient quantities through diet (Danulat and Hochachna, 1989). Creatine level did not vary much

due to starvation up to 6 weeks (Danulat and Hchachka, 1989). Danulat and Hochachka (1989) have have also suggested that the difference between creatine level in flounder and trout may reflect differences in swimming activity or nutritional status.

According to Yang *et al.* (2003) the creatine level in carp normally ranges from 0.47 mg/dl to 0.53 mg/dl under normal condition but could be increased up to 0.93 mg/dl to 1.03 mg/dl under stressed condition. Plasma creatine levels can be used as rough indicators of glomerular filtration rate and kidney functions (Maita *et al.*, 1984). Low levels of createnine and uric acid have no significance but increasing their values indicates several disturbances in renal system (Maxine and Benjamin, 1985). Increased level of creatine is also an indicator of kidney damage, muscular dystrophy and physical excretion of organism (Masopust, 1998).

2.1.3.1.2.7. Serum enzymes

Plasma /serum enzymes are broadly classified into two categories (a) plasma specific enzymes, having function and purpose in the blood; and (b) plasma non-specific enzymes that are derived from moribund cells (Gowenlock, 1988). Measurement of the activities of the plasma non-specific enzymes has diagnostic potential in fish toxicology and pathology because enzyme activities can often be related to cell damage in specific organ. For example the liver is rich in Glutamic oxaloacetic transaminase (GOT) and Glutamic-pyruvate transaminase (GPT) and changes in plasma levels of these enzymes are indicative of liver dysfunction (Racicot *et al.*, 1975).

There are also methodological considerations that can influence the interpretation of plasma enzyme activities. In rainbow trout, the method of blood sampling influenced the activity of at least two plasma non-specific enzymes: Lactate dehydrogenase (LDH), and Creatine phosphokinase (CPK). These enzymes which are abundant in skeletal muscle are greater in blood sample via caudal puncture than via cardiac puncture.

The increment of Aspertate amino transferase (AST) and Alanine amino transferase (ALT) often related to activity of liver. Fish liver is a major organ involved in metabolic process (Pacheco and Santosh, 2001) e.g. glucose utilizing, glucose producing and glucose storing (Lerman *et al.*, 2004). The increased ALT and AST value in fish reveals enzymes exporting from liver into blood stream (Yang and Chen, 2003; and Perez-Rostro *et al.*, 2004). This implies increase energy consumption in fish (Fernandez, 1989) and also indicates hyper hepatic metabolism (Barcellos *et al.*, 2003).

AST and ALT are frequently used in diagnosis of damage caused by pollutants in various tissues such as liver, muscle and gills (De la Tot *et al.*, 2000). It is generally accepted that increased activity of these enzymes in extracellular fluid of plasma is a

sensitive indicator of even minor cellular damage in the liver of the fish. AST and ALT are quantitatively important in transmission of amino acids, thereby, allowing interplay between carbohydrate and protein metabolism during the fluctuating energy demand of the organisms in various adaptive situations (Verma *et al.*, 1981). Moreover, Palikova *et al.* (2010) stated that ALT and AST are most frequently tested .enzymes in fish or indication of cyanobacterial toxicity of microcystins.

Lactate dehydrogenase (LDH) is mainly used to detect tissue alterations and as an aid in the diagnosis of anaemia, gill and liver disease (Banaee *et al.*, 2008). Most authors reported increased plasma LDH concentration in various fishes e.g. *Anguilla* (Ceron *et al.*, 1997 and Sancho *et al.*, 2000), *Puntius conchonius* (Gill *et al.*, 1990), *Heteropneustes fossilis* (Singh and Srivastava, 1982) and *Channa striata* (Natarajan, 1989) following acute effects of organophosphorous pesticides including diazinon. The levels of LDH in *Labeo rohita* were increased with cypermethrin (Philip *et al.*, 1995 and Das and Mukherjee, 2003).

According to Rehulka (2002) the plasma AST, ALT and LDH level for rainbow trout were 13.74 µkat/l, 1.67 µkat/l 24.90 µkat/l, respectively. According to Jin Ha Yu (2010) when *Silurus asotus* were subjected to infestation of *Edwardsiella tarda,* the AST value increased from 120 U/l to 600 U/l. Paurgholam *et al.* (2013) found that when grass cap (*Ctenopharyngodon idella*) is challenged with *Aeromonas hydrophila* following exposure to sub lethal concentration of diazinon, for 30 days the ALT value increased from 5.4 µ/L to 7.2 µ/L, AST value increased from 44 µ/L to 59.5 µ/L and LDH value decreased from 1625.5 µ/L to 1121.5 µ/L.

2.1.3.1.2.8. Cortisol

Cortisol is the principal corticoid secreted by the inter-renal tissue (steroidogenic cells) located at the head kidney of teleosts fish (Iwama *et al.*, 1999). Hormone is released by the activation of the hypothalamus pituitary inter-renal axis (HPI axis) (Mommsen *et al.*, 1999), When an organism undergoes stress conditions, the hypothalamus releases corticotrophin releasing factor (CRF) towards blood circulation. This polypeptide further stimulates secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland (Fryer and Lederis, 1986), which finally activates the release of cortisol by the inter-renal tissue (Mommsen *et al.*, 1999). Plasma cortisol (nmol/l) values of different species of fish before and after stressed is given in Table 3. Dynamics of Cortisol and Catecholamine in the production of glucose is represented in Figure 2.

In experiments of acute stress (Davis *et al.*, 2006), cortisol reached highest concentration after 1 hour of stress and returned to basal level after 6 hour (Iwama *et al.*, 2006). It has been suggested that after stress, the cortisol levels of fish returns to basal level to avoid tissue damage (Wendelaar Bonga, 1997). Common dentex (*Dentex dentex*)

Table 3. Plasma cortisol (nmol/l) values of different species of fish before and after stress

Species	Stressor	Prestress	Poststress	Exposure	References
Atlantic salmon (*Salmo salar*)	Sea lice challenge	99	339	Chronic	Bowers *et al.* (2000)
Atlantic salmon (*Salmo salar*)	Confinement	27	151	Acute	Sadler *et al.* (2000)
Atlantic salmon (*Salmo salar*)	Confinement	27	124	Acute	Sadler *et al.* (2000)
Brook trout (*Salvelinus fontinalis*)	Handling and confinement	19	242	Acute	Benfey and Biron (2000)
Brook trout (triploid) (*Salvelinus fontinalis*)	Handling and confinement	2	146	Acute	Benfey and Biron (2000)
Pallid sturgeon (*Scaphirhynchus albus*)	Confinement	5	16	Acute	Barton *et al.* (2000)
Pallid sturgeon (*Scaphirhynchus albus*)	Handling	5	8	Acute	Barton *et al.* (2000)
Rainbow trout (*Oncorhynchus mykiss*)	Handling and confinement	77	698	Acute	Benfey and Biron (2000)
Sea bream (*Sparus aurata*)	Crowding	13	358	Chronic	Ortuño *et al.* (2001)
Rainbow trout female (*Oncorhynchus mykiss*)	Trapping	57	764	Acute	Clements *et al.* (2002)
Rainbow trout (*Oncorhynchus mykiss*)	Trapping	16	380	Acute	Clements *et al.* (2002)
Walleyes (*Stizostedion vitreum*)	Capture and transport	33-315	380-480	Acute	Barton *et al.* (2003)

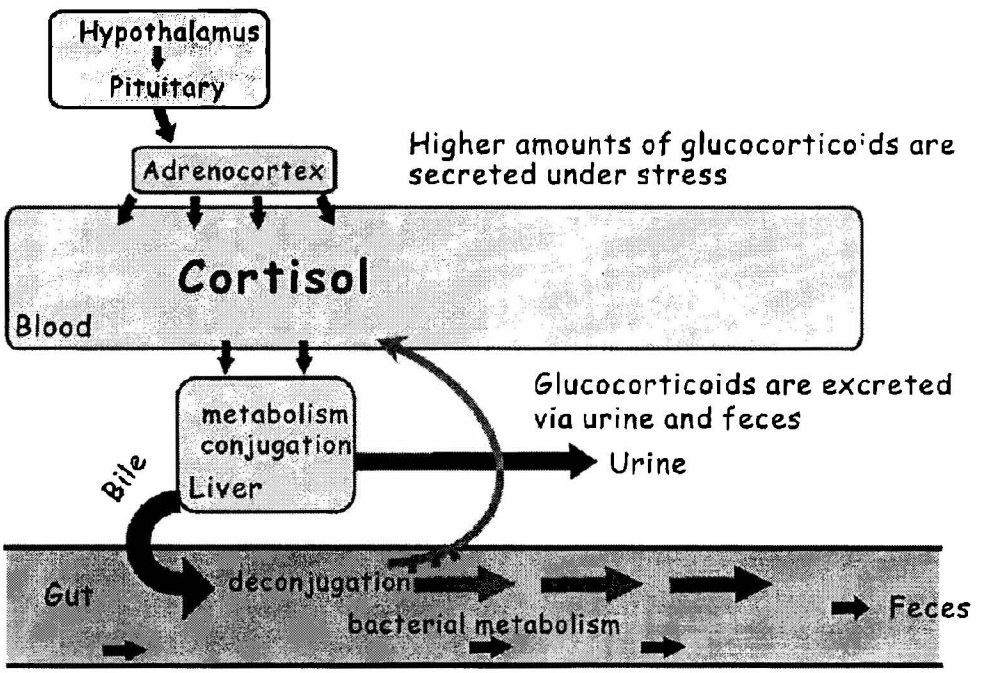


Figure 2. Dynamics of Cortisol and Catecholamine in the production of glucose where (+) sign indicates positive modulation and (-) sign indicates negative modulation. ((Moberg1985,1992)

increased its cortisol levels immediately after handling and then returned to the basal level after 8 hours (Moralles *et al.*, 2005). Carp (*Cyprinus carpio*) increased plasma cortisol when retained in anglers-keep nets but returned to basal levels within 4 hours (Pottinger, 1998).

Cortisol is widely used both as long term and short term stress index, even if it may be influenced by species, feeding reproductive cycles, seasonal cycles, photoperiod, husbandry condition and sampling. (Pickering and Potinger, 1983; Audet *et al.*, 1986; Pickering *et al.*, 1987; Lowe *et al.*, 2005; Vijayan and moon., 1994; Pickring and Pottinger, 1998; Reddy *et al.*, 1995; Thorpe *et al.*, 1897; Wandellar Bonga, 1997; Barton, 2002).

Though cortisol is used primarily as an endocrine response and as an indicator of stressor in aquaculture system, it has some multiple functional role including liver metabolism, immune function and osmoregulation. It is a predictable indicator of magnitude and duration of acute stress (Pickering and Potinger, 1982).

Resting levels as low as 5 ng/ml have been reported for salmonids by Pickering *et al.* (1985), although studies on other teleosts (Peter *et al.*, 1978; Lamba *et al.*, 1983; Venkatesh *et al.*, 1989), reported higher levels ranging between 10 ng/ml and 50 ng/ml. In metabolically active species such as salmonids and cyprinids, cortisol elevations as high as 400-600 ng/ml have been reported while species of intermediate activity such as largemouth bass (*Micropterus salmonids*) had levels around 200 ng/ml and inactive species such as the gar (*Lepiososteus* sp) and Bowfin *(Amia calva)* had peaks around 50-60 ng/ml (Davis and Parker, 1990).

CHAPTER-3

Materials & Methods

3. MATERIALS AND METHODS

3.1. Experimental site

All the experiments were carried out in the wet and post-graduate laboratories of the Department of Fisheries Resource Management, Faculty of Fishery Sciences, Chakgaria, Panchasayar, Kolkata 700094, India (Lat. 22° 82'N; Long. 88° 20'E).

3.2. Experimental fish

Healthy and active advanced fingerlings of rohu (*Labeo rohita*) (Plate 1) were used for the experiments. The fish were procured from Naihati fish market, North 24 Parganas, West Bengal and transported in double layer oxygen packed polythene bags to the wet laboratory of the Department. They were then transferred to Fiberglass Reinforced Plastic (FRP) tanks (500 L) filled with tap water (chlorine free) and left undisturbed whole night. The stock was acclimated under well aerated conditions. During the acclimation period the fish were fed with commercial feed MFC (Mohan Feeds and Chemicals) containing 32% protein @ 3% of the body weight, twice a day. Manual water exchange (20-25%) was done daily after siphoning out the left out feed and excreta. Tap water from the same source was used in all experiments.

3.2. Stressors

The physiological effects of acute stressors viz., (i) handling – short term and long term (ii) transportation and (iii) experimentally challenged with bacteria were assessed.

3.3. Chemicals and glasswares

The glasswares used throughout the experiment were of Borosil, Riviera and Scott Duran makes. Chemicals of various makes viz. Sigma, SRL, HiMedia and Merck were used in the present investigation.

3.4. Experimental protocol

3.4.1. Handling stress

3.4.1.1. Acute short term (single handling) stress

Acute short term (single handling) stress experiment was carried out on 27.05.2013. Six numbers of uniform sized (18.50 – 19.50 cm) acclimated rohu (*Labeo rohita*) were transferred to each pre-cleaned, dried and well aerated experimental glass aquaria of 50L capacity, filled with 30L of water. The upper side of the aquaria were completely covered with net piece and tied

with the help of rubber belt to avoid jumping of fishes. Four experimental groups in duplicate were made namely - one *control (C) (undisturbed/ unstressed)* and three *acute single handling stress groups* (ASHS1, ASHS2 and ASHS3). The fish were maintained for seven days in aquaria, during which they were fed at 3% body weight with the same feed as used during acclimation. Manual water exchange (20-25%) was done daily after siphoning out the left out feed and excreta. The fish were starved and left undisturbed for 24h prior to experiment. Stress groups consisted of the fishes subjected to acute handling (Plate 2) (chasing, netting and releasing back) for 5 min (ASHS1), 10 min (ASHS2) and 15 min (ASHS3). The fish from each group were caught by scoop net and blood samples collected as described in section 3.4.3 for further analysis.

3.4.1.2. Acute long term stress

Acute long term stress study was performed for seven days from 27.05.2013 to 03.06.2013. Six numbers of uniform sized (18.50 – 19.50 cm) acclimated rohu (*Labeo rohita*) were transferred to each pre-cleaned, dried and well aerated experimental glass aquaria of 50L capacity, filled with 30L of water. The upper side of the aquaria were completely covered with net piece and tied with the help of rubber belt to avoid jumping of fishes. Four experimental groups in duplicate were made namely - one *control* (C) *(undisturbed/ unstressed)* and three *acute long term handling stress groups* (ALHS1, ALHS2 and ALHS3). The fish were maintained for seven days in aquaria, during which they were fed at 3% body weight with the same feed as used during acclimation. Manual water exchange (20-25%) was done daily after siphoning out the left out feed and excreta. The fish were starved and left undisturbed for 24h prior to experiment. Stress groups consisted of the fishes subjected to acute long term handling (chasing, netting and releasing back) for 5 min (ALHS1), 10 min (ALHS2) and 15 min (ALHS3) on daily between 5.30 pm and 6.00 pm for seven days. The feeding and maintenance was continued during this period also as described in section 3.4.1.1. The fish from each group were caught by scoop net and blood samples collected as described in section 3.4.3 on the termination day of experiment for further analysis.

3.4.2. Transportation stress

The transportation stress response and changes in biochemical and haematological indices were investigated in advanced fingerlings (14.00 – 15.00 cm) of rohu (*Labeo rohita*) transported from Naihati to the Department on 17.06.2013 and 18.06.2013. About 10 fish were caught from the rearing pond of a local hatchery in Naihati, and held in hapa for 24h without feeding. The first blood sample from this group was taken as described in section 3.4.3 at the

Plate 1. Experimental fish: Rohu (*Labeo rohita*)

Plate 2. Fish subjected to acute handling stress

hatchery itself before they were taken to market and marked as BD (before disturbance), which was taken as control. The fish in the rearing pond were also starved for 24h before transportation. The pond fish were caught for further transportation. The second blood sample (Plate 4) was taken after they were transported to the Naihati fish market (10 min from hatchery) by placing them in an open large aluminum container, locally called '*handi*', (Plate 3) (100 L capacity; filled with 40% water and placed with 30 kg fish/handi) on a tricycle and this group was marked as BT (before transportation).

The fish were then packed in sealed double layer oxygen packed polythene bags (30L) (Plates 5 and 6) containing one fourth of water and three fourth of oxygen and transported for 2.5 h in a motor vehicle from Naihati fish market, North 24 Parganas to the Department of Fisheries Resource Management, Faculty of Fishery Sciences, Chakgaria. The fish were transported at three stocking densities, viz., 500 g/bag, 1 kg/ bag and 1.5 kg/ bag in duplicate. The third blood samples were taken immediately after transportation from each bag separately and were marked as AT1, AT2 and AT3 (immediately after transportation) for stocking densities of 500 g/bag, 1 kg/ bag and 1.5 kg/ bag, respectively.

3.4.3. Bacteriological stress

The study on bacteriological stress was carried out for 7 days between 30.06.2013 and 07.07.2013. Twelve numbers of uniform sized (13.00 – 14.00 cm) acclimated rohu (*Labeo rohita*) were transferred to each pre-cleaned, dried and well aerated experimental glass aquaria of 50L capacity, filled with 30L of water. The upper side of aquaria were completely covered with net piece and tied with the help of rubber belt to avoid jumping of fishes. The fish were maintained for seven days in aquaria, during which they were fed at 3% body weight with the same feed as used during acclimation. Manual water exchange (20-25%) was done daily after siphoning out the left out feed and excreta. The fish were starved and left undisturbed for 24h prior to experiment. Two bacterial fish pathogens, viz., *Aeromonas hydrophila* (N10P) and *Edwardsiella tarda* (CGH9) exhibiting LD_{50} values $2.37x10^8$ cells/fish and $3.16x10^8$ cells/fish, respectively, obtained from the Department of Aquatic Animal Health, Faculty of Fishery Sciences, were injected (intramuscular) (Plate 7) to evaluate the stress response in terms of haematological and biochemical changes. The sub-lethal doses used in the present study were 2.40×10^7 cells of *Aeromonas hydrophila*/fish and 1.70×10^7cells of *Edwardsiella tarda*/fish. The fish in control tank were injected with 0.1 ml of physiological saline. Experiment was carried out in duplicate. The blood samples were taken after 6h as well as after 7 days of post-injection

Plate 3. Transportation of fish in '*handi*' from hatchery to Naihati fish market

Plate 4. Blood sample collection before transportation

Plate 5. Oxygen packing before transportation

Plate 6. Packing of fish in double layered polythene bag

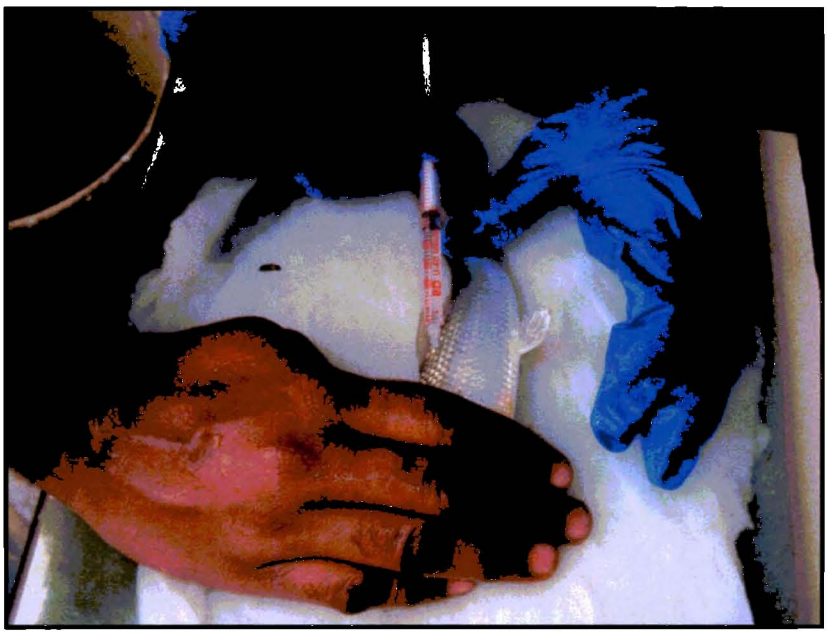


Plate 7. Intramuscular inoculation of *Aeromonas hydrophila*

from each aquarium. The feeding and maintenance was continued during this period also as described in section 3.4.1.1.

3.4.3. Collection of blood and serum samples

(a) Blood collection: In order to minimize the possible variations in blood parameter values, the technique was standardized as follows and same technique was used for collection of blood samples in all the experiments. The fish were sampled from the experimental aquaria using scoop net with minimum handling stress and transferred into plastic buckets containing water of the same temperature and immediately anaesthetized with clove oil (50µL/L). The blood from the experimental fishes was drawn (Plates 8a and 8b) using 1 ml sterile disposable plastic insulin syringes (30G) through cardiac puncture. The use of plastic syringes is a necessary precaution with fish blood because contact with glass results in shortened coagulation times (Smith *et al.*, 1952). The anticoagulant used was tri-sodium citrate (3.8% w/v) and blood samples were collected in 1.5 ml eppendorf tubes rinsed with anticoagulant to prevent coagulation and used for the estimation of red blood corpuscles (RBC), white blood corpuscles (WBC), haemoglobin and haematocrit values.

(b) Serum collection: The blood was also collected by syringe without anticoagulant and placed in 1.5 ml eppendrof tube (Plates 9a 9b) and kept overnight in a refrigerator in a slanting position; while the blood clots giving a straw coloured supernatant at the top. Then it was centrifuged at 4000 rpm for 4 min to get the clear serum at the top. The supernatant was collected by micro centrifuge to another eppendroff tube and stored in deep freeze (-20^{0}C) for further analysis of biochemical parameters.

3.4.4. Analysis of blood and serum samples

3.4.4.1. Haematological parameters

3.4.4.1.1. Total Erythrocytes Count (TEC)

The total erythrocytes count (TEC) or Red Blood Corpuscles (RBC) of fish blood was made in Neubauer's counting chamber using RBC diluting fluid (Dacie's fluid) (Annexure 1). Twenty microlitre (µl) of the collected blood sample from the eppendroff tubes after thorough shaking was sucked into a micropipette and immediately diluted to 3980 µl of RBC diluting fluid (Plate 10) in a test tube (1:200 ratio). The diluted blood was mixed by tilting the tube gently avoiding destruction of the cells. Some of the diluted blood solution was drawn into the micropipette and expelled out touching the tip of the pipette to the edge of the cover slip on the

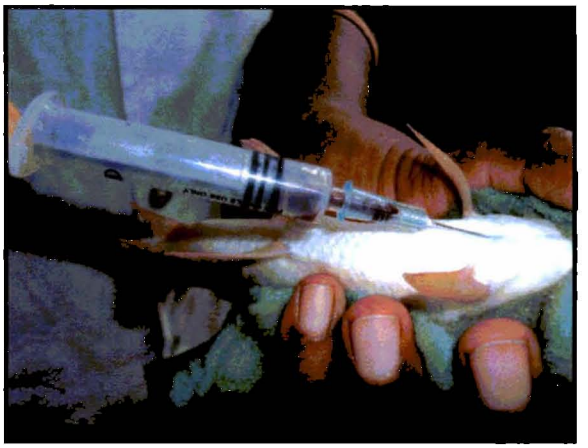


Plate 8(a)

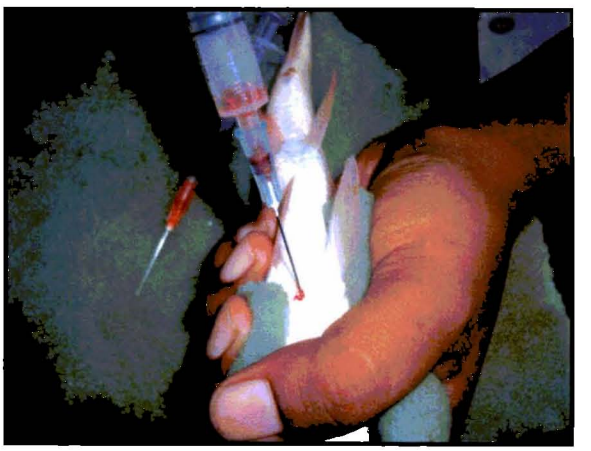


Plate 8(b)

Plates 8(a) and 8(b). Collection of blood for haematological and biochemical analyses

Plate 9(a)

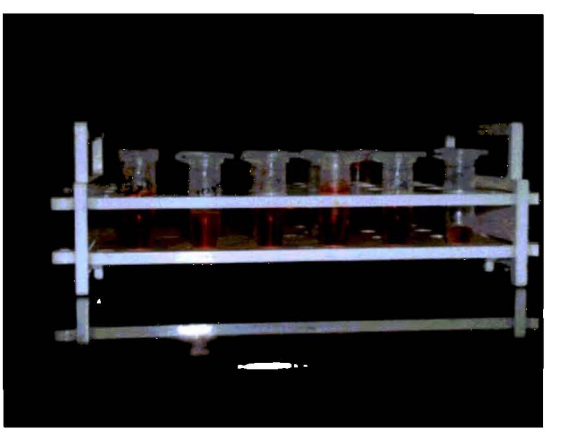


Plate 9(b)

Plates 9(a) and 9(b). Collection of serum for haematological and biochemical analyses

Plate 10. Estimation of red blood corpuscles

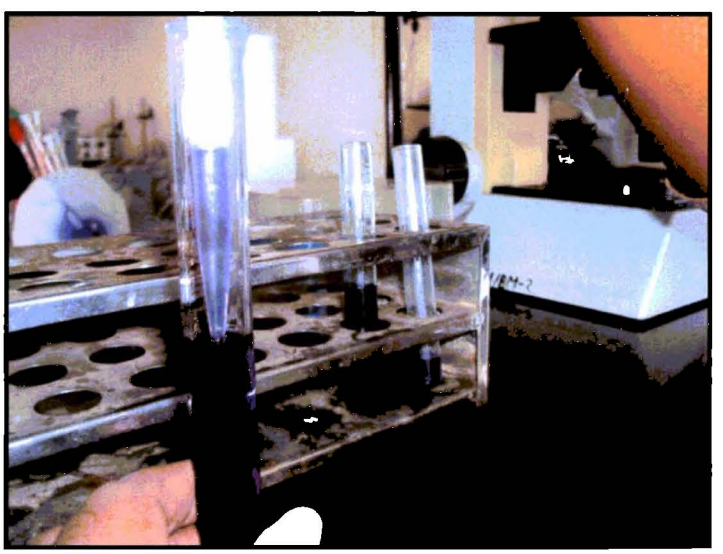


Plate 11. Estimation of white blood corpuscles

counting chamber. The capillary action will draw the diluted blood into the chamber. Then counting was done under binocular research microscope (40X) (Olympus iNEA). The cells occurring in five small squares at the centre of the grid with a total area of 0.02 mm^3 (1/50 of 1mm^3) were counted for erythrocytes. The dilution was 1:200; therefore the number of cells occurring per mm^3 was calculated as follows:

$$\text{Number of cells occurring per mm}^3 = \frac{\text{Number of cells counted in 5 small squares X 200 (dilution)}}{\text{0.2 (total area counted)} \times \text{0.1(depth of the fluid)}}$$

3.4.4.1.2. Total Leukocytes Count (TLC)

The total leukocyte count (TLC) or White blood corpuscles (WBC) of fish blood was made in Neubauer's counting chamber using Shaw's WBC diluting fluid (Shaw's WBC diluting fluid: Annexure – 2) (Plate 11). The dilution procedure was the same as mentioned in RBC counting procedure. The numbers of cells were counted in four large squares (1 mm^3 each) under binocular research microscope (40X) (Olympus iNEA). The total leukocyte count was expressed as follows.

$$\text{Number of cells occurring per mm}^3 = \frac{\text{Number of cells counted in 0.1 mm}^3 \text{ X 200 (dilution)}}{\text{4 (total area counted)} \times \text{0.1 (depth of the fluid)}}$$

3.4.4.1.3. Haematocrit (Ht)

The haematocrit (Ht) value was determined by microhaematocrit centrifugation (Plate 12). The haematocrit tubes (HiMedia, 7 cm x 11 mm) were taken and 2/3rd of their total lengths were filled with blood by capillary attraction and surface tension and one end of the tubes were sealed with plasticine. The capillary tubes were placed in to the grooves of the centrifuge, so that the sealed end was away from the center. The centrifugation was done at 10,000 rpm for 10 min. The tubes were then taken out and the haematocrit value (Ht) was calculated as given below and expressed as the percentage fraction of blood cells in the total volume.

$$\text{Haematocrit value (Ht)(expressed in \%)} = \frac{\text{Height of R. B. C. column}}{\text{Total height of the column}} \times 100$$

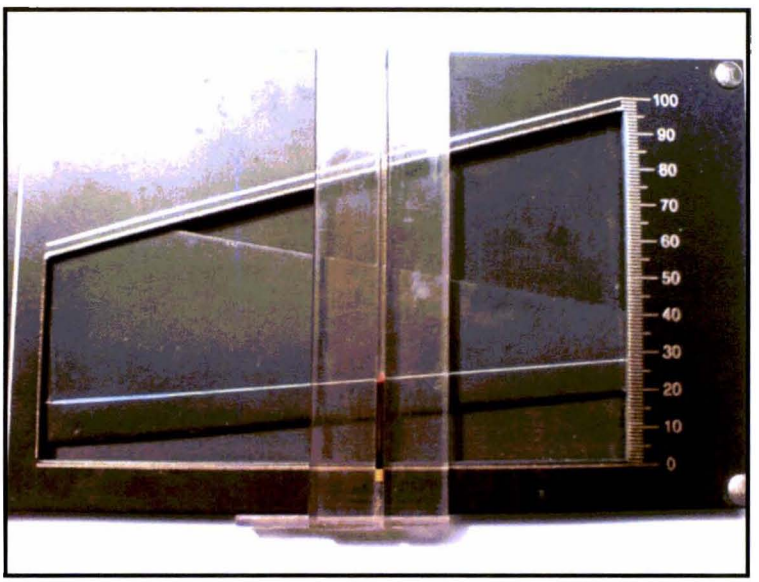


Plate 12. Haematocrit measurement

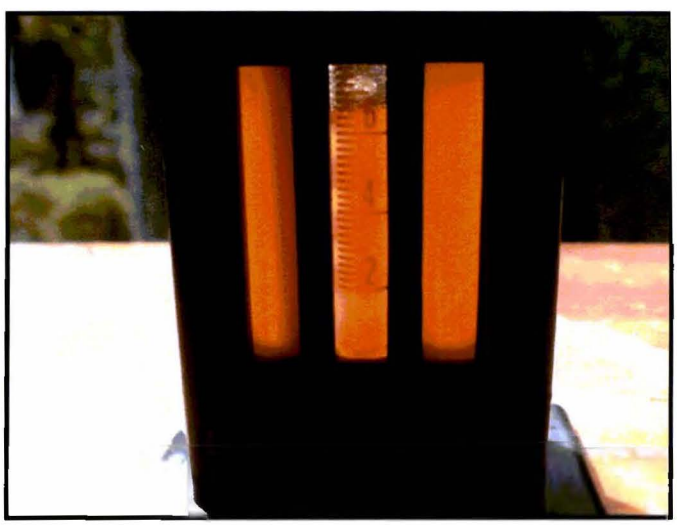


Plate 13. The haemoglobin estimation using Sahli's haemoglobinometer

3.4.4.1.4. Hemoglobin concentration (Hb)

The haemoglobin concentration in percentage was determined by acid haematin method using Sahli's haemoglobinometer (Plate 13). The graduated tube was filled with 1/10 N HCl up to the lowest mark, i.e., 10. Then 20 µl of blood was transferred to the tube and mixed properly by the stirring rod and kept stand for 15 min. The colour developed by the reaction of hemoglobin with dilute acid, producing acid-haematin, was matched colourimatrically with that of the standard by slowly and drop-wise adding distilled water or 1/10N HCl. When the colour matched, the reading was taken immediately as hemoglobin % or g/dl.

3.4.4.2. Biochemical parameters

3.4.4.2.1. Serum glucose

The serum glucose was determined by using glucose test kit (Span Diagnostics Ltd.) following GOD-POD method. To 1500 µl of the reagent solution [containing phosphate buffer (pH 6.50), 100 mmol/L, GOD ≥15000 U/L, POD ≥1600 U/L, 4-AAP 0.28 mM/L, phenol preservative 10 mmol/L] taken in a clean and dry test tube, 20 µl of serum was added. Simultaneously, a standard, using standard glucose (100 mg/100 ml), and a blank (only reagent solution) were prepared (1500 µl each). The contents in each test tube were mixed thoroughly and incubated at room temperature for 10 min. Then the absorbance of the contents were taken at wavelength of 505 nm using Photometer (Model: 5010 v5+, Robert Riele KG, Berlin) after zeroing it with blank. Final glucose concentration was found by putting the absorbance values (optical density) into standard formula and expressed as mg/dl.

3.4.4.2.2. Total serum protein

The total serum protein was determined by using protein test kit (Autopak Diagnostics Ltd.) following Biruet method. To 1000 µl of reagent solution (containing sodium hydroxide 3.8mol/L, pottasium sodium tartrate 0.1mol/L, cupric sulphate 33 mmol/L, potassium iodide 30 mmol/L, surfactant 20g/L) taken in a clean and dry test tube 10 µl of serum was added. Simultaneously, a standard, using standard protein (BSA 60 g/L) and a blank (only reagent solution) were prepared (1000 µl each). The contents in each test tube were mixed thoroughly and incubated at room temperature for 20 min. Then the absorbance of the contents were taken at wavelength of 505 nm using Photometer (Model: 5010 v5+, Robert Riele KG, Berlin) after zeroing it with blank. Final protein concentration was found by putting the absorbance values (optical density) into standard formula and expressed as g/dl.

3.4.4.2.3. Serum triglycerides

The triglyceride was determined by using triglyceride test kit, (Span Diagnostics Ltd.) following GPO-PAD, end point assay method. Ten µl serum sample was taken and serum triglycerides were measured at room temperature by following the kit procedure through Photometer (Model: 5010 v5+, Robert Riele KG, Berlin; Plate 14). The triglycerides concentration was calculated by putting the absorbance values as 505 nm (optical density) into standard formula and expressed as mg/dl.

3.4.4.2.4. Serum urea

The urea was determined by using urea test kit (Span Diagnostics Ltd.) following Urease Berthelot, end point assay method. Ten µl serum sample was taken and serum urea was measured at room temperature by following the kit procedure through Photometer (Model: 5010 v5+, Robert Riele KG, Berlin). The urea concentration was calculated by putting the absorbance values as 578 nm (optical density) into standard formula and expressed as mg/dl.

3.4.4.2.5. Serum creatinine

The creatinine was determined by using creatinine test kit (Span Diagnostics Ltd.) following Modified Jaffe's Reaction, Initial Rate Assay, method. One hundred µl serum sample was taken and serum creatinine was measured at room temperature by following the kit procedure through Photometer (Model: 5010 v5+, Robert Riele KG, Berlin). The creatinine concentration was calculated by putting the absorbance values as 505 nm (optical density) into standard formula and expressed as mg/dl.

3.4.4.2.6. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

The ALT and AST were determined by using ALT and AST test kits (Span Diagnostics Ltd.) following Modified UV (IFCC), and kinetic assay method. One hundred µl serum sample was taken and serum ALT and AST were measured at room temperature by following the kit procedure through Photometer (Model: 5010 v5+, Robert Riele KG, Berlin). The ALT and AST concentrations were calculated by putting the absorbance values as 340 nm (optical density) into standard formula and expressed as IU/L.

Plate 14. Photometer 5010 v5+ (Robert Riele KG, Berlin) used for biochemical analyses

3.4.4.2.7. Serum Lactate dehydrogenase (LDH)

The LDH was determined by using LDH (P-L) test kit (Crest Biosystem) following modified IFCC method. Twenty µl serum sample was taken and serum LDH was measured at room temperature by following the kit procedure through Photometer (Model: 5010 v5+, Robert Riele KG, Berlin). The LDH concentration was calculated by putting the absorbance values as 340 nm (optical density) into standard formula and expressed as U/L.

3.4.4.2.8. Cortisol

To estimate cortisol levels in fish serum, the cortisol test EIA kit (AccuBind Elisa Microwells, Cortisol test system) was procured from Monobind Inc, Lake Forest, USA. The procedure follows the basic principle of enzyme immunoassay, where there was competition between unlabeled antigens for a fixed number of specific monoclonal anticortisol antibody binding sites. The amount of enzyme-labeled antigen bound to the antibody was inversely proportional to the concentration of the unlabeled analyzer present. Unbound materials were removed by decanting and washing the well. One hundred µl TMB chromogen solution to each well was added and incubated at room temperature for 15 min and as well as in dark condition. After that a strong acidic (1N HCl) stopping solution was then added and the degree of enzymatic turnover of substrate was determined by dual wavelength absorbance measurement at 450 and 620 nm. The serum cortisol level was expressed in µg/dl serum.

3.4.5. Analysis of water quality parameters

Important water quality parameters like temperature using thermometer (N.S. Dimple thermometer -305 MM), pH using pH paper (HiMedia) and dissolved oxygen by following Winkler's method (APHA, 1998) were measured after taking the blood samples from each experimental aquarium.

3.4.6. Statistical analysis

The results of haematological and biochemical indices are expressed as mean$\pm$ standard deviation throughout. Student's two tailed t-test (paired design) was used to test the significant differences ($P<0.05$) among stressed groups as well as between stressed and unstressed (control) groups (Snedecor and Cochran, 1980).

Table 4. Summary of haematological and biochemical indices analyzed

Parameters analyzed	Sample	Instruments used	Kit or chemicals used
Total erythrocyte count (TEC) or red blood corpuscles (RBC)	Blood	Neubauer's counting chamber	Dacies RBC diluting fluid
Total leukocyte count (TLC) or whitw blood corpuscles (WBC)	Blood	Neubauer's counting chamber	Shaw's WBC diluting fluid
Hemoglobin	Blood	Sahli's haemoglobinometer	1/10 N HCl
Haematocrit	Blood	Micro centrifuge and Haemometer scale	
Glucose	Serum	Photometer 5010 v5+ (Robert Riele KG, Berlin)	Glucose test kit, GOD-POD, End point assay and Kinetic assay; Span Diagnostics Ltd.
Total serum protein	Serum	Photometer 5010 v5+ (Robert Riele KG, Berlin)	Total protein kit, Biuret method; Siemens Ltd; Autopak Diagnostics Ltd.
Triglycerides	Serum	Photometer 5010 v5+ (Robert Riele KG, Berlin)	Triglycerides test kit.GPO-PAP, End point assay; Span Diagnostics Ltd.
Urea	Serum	Photometer 5010 v5+ (Robert Riele KG, Berlin)	Urea test kit, Urease Berthelot, End point assay; Span Diagnostics Ltd.
Creatinine	Serum	Photometer 5010 v5+ (Robert Riele KG, Berlin)	Creatinine test kit, Modified Jaffe's Reaction, Initial rate assay; Span Diagnostics Ltd.
Alanine amino-transferase (ALT)	Serum	Photometer 5010 v5+ (Robert Riele KG, Berlin)	ALT test kit, Modified UV (IFCC), Kinetic assay; Span Diagnostics Ltd.
Aspartate amino-transferase (AST)	Serum	Photometer 5010 v5+ (Robert Riele KG, Berlin)	AST test kit, Modified UV (IFCC), Kinetic assay; Span Diagnostics Ltd.
Lactate Dehydrogenase (LDH)	Serum	Photometer 5010 v5+ (Robert Riele KG, Berlin)	LDH (P-L) test kit, MOD. IFCC method, Crest biosystem
Cortisol	Serum	Photometer 5010 v5+ (Robert Riele KG, Berlin)	Cortisol test EIA kit AccuBind Elisa Microwells, Cortisol test system, Product Code: 3625-300. Monobind Inc. Lake Forest, USA

CHAPTER-4

Results

4. RESULTS

In the present study haematological and biochemical changes in rohu (*Labeo rohita*) subjected physical and biological stressors were analysed and the results obtained are presented below.

4.1. Acute single handling stress

Variations in haematological and biochemical parameters of *Labeo rohita* subjected acute single handling stress are presented in Table 5.

4.1.1. Haematological parameters

4.1.1.1. Total erythrocyte count (TEC) or Red blood corpuscles (RBC)

The mean total erythrocyte count in the unstressed fish (control) was lower ($1.15\pm0.05\times10^6$ no/ml) and increased due to handling procedure. The TEC values were found to be $1.30\pm0.00\times10^6$ no/ml, $1.45\pm0.05\times10^6$ no/ml and $2.00\pm0.01\times10^6$ no/ml when fish were subjected to 5 min (ASHS1), 10 min (ASHS2) and 15 min (ASHS3) acute single handling stress, respectively (Table 5; Figure 3).

4.1.1.2. Total leukocyte count (TLC) or White blood corpuscles (WBC)

In the present study, the mean total leukocyte count in the unstressed fish (control) was lower ($51.50\pm0.05\times10^3$ no/ml) which showed an increase trend with acute and chronic handling duration. The mean TLC values in rohu subjected to 5 min (ASHS1), 10 min (ASHS2) and 15 min (ASHS3) acute single handling stress were found to be $85.00\pm02.00\times10^3$ no/ml, $91.50\pm0.05\times10^3$ no/ml and $99.00\pm2.00\times10^3$ no/ml, respectively (Table 5; Figure 4).

4.1.1.3. Haemoglobin

In the unstressed (control) fish haemoglobin content was 3.80±0.20 g/dl. Maximum haemoglobin content (4.10±0.00 g/dl) was recorded in ASHS3 (fish subjected to 15 min acute single handling stress) where as minimum value (3.10±0.10 g/dl) was found to be in ASHS1 (fish subjected for 5 min acute single handling stress) (Table 5; Figure 5). An intermediate value of 3.80±0.20 g/dl was noticed in ASHS2 (fish subjected to 10 min acute single handling stress).

4.1.1.4. Haematocrit

The mean haematocrit value in the unstressed (control) fish was comparatively lower (14.20±0.30 %) and increased with the duration of acute single handling stress, i.e. it was 22.00±1.00 % for 5 min (ASHS1), 24.50±1.10 % in the case of 10 min (ASHS2) and 26.25± 0.75 % in the case of 15 min (ASHS3) (Table 5; Figure 6).

Table 5. Haematological and biochemical variations (mean ± standard deviation) in *Labeo rohita* subjected acute single handling stress (ASHS) (n=6)

Parameter	Unstressed	Stressed		
	(Control) (C)	ASHS1 (5 min)	ASHS2 (10 min)	ASHS3 (15 min)
Total erythrocyte count (TEC) or Red blood corpuscles (RBC) ($x10^6$ no/ml)	1.15 ± 0.05^{a}	1.30 ± 0.00^{ac}	1.45 ± 0.05^{ac}	2.00 ± 0.01^{b}
Total leukocytes count (TLC) or White blood corpuscles (WBC) ($x10^3$ no/ml)	51.50 ± 0.50^{a}	85.00 ± 2.00^{b}	91.50 ± 0.50^{bce}	99.00 ± 2.00^{de}
Haemoglobin (Hb) (g/dl)	3.80 ± 0.20^{a}	3.10 ± 0.10^{a}	3.80 ± 0.20^{a}	4.10 ± 0.00^{b}
Haematocrit (Ht) (%)	14.20 ± 0.30^{a}	22.00 ± 1.00^{bc}	24.50 ± 1.50^{ce}	26.25 ± 0.75^{de}
Serum glucose (mg/dl)	30.00 ± 1.00^{a}	45.00 ± 1.00^{b}	61.00 ± 1.00^{c}	74.50 ± 0.50^{d}
Serum total protein (g/dl)	2.55 ± 0.05^{a}	2.39 ± 0.00^{a}	2.45 ± 0.05^{a}	2.23 ± 0.16^{a}
Serum tryglycerides (mg/dl)	24.00 ± 2.00^{a}	41.00 ± 0.00^{b}	43.00 ± 1.00^{b}	46.50 ± 1.50^{bc}
Serum urea (mg/dl)	3.95 ± 0.50^{a}	5.60 ± 0.60^{a}	6.60 ± 0.60^{a}	7.00 ± 1.00^{a}
Serum creatinine (mg/dl)	0.36 ± 0.03^{a}	0.35 ± 0.01^{a}	0.39 ± 0.01^{a}	0.40 ± 0.02^{a}
Serum alanine aminotransferase (ALT) (IU/L)	10.00 ± 1.00^{a}	28.00 ± 2.00^{b}	28.00 ± 1.00^{bc}	36.00 ± 0.00^{bc}
Serum aspartate aminotransferase (AST) (IU/L)	41.00 ± 2.00^{a}	56.50 ± 3.50^{b}	58.00 ± 2.00^{b}	60.00 ± 2.00^{c}
Serum cortisol (µg/dl)	20.00 ± 2.00^{a}	30.00 ± 1.00^{b}	53.00 ± 2.00^{bc}	56.00 ± 0.00^{c}

***The values with different superscript differ significantly (P<0.05)**

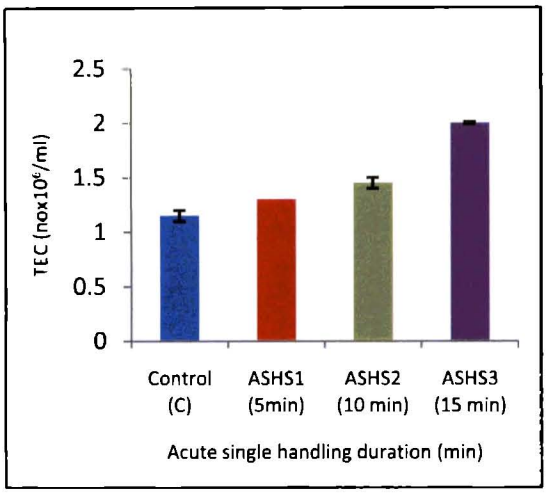


Figure 3

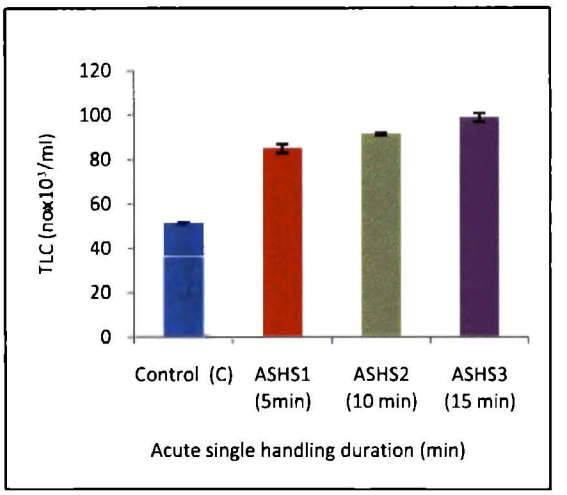


Figure 4

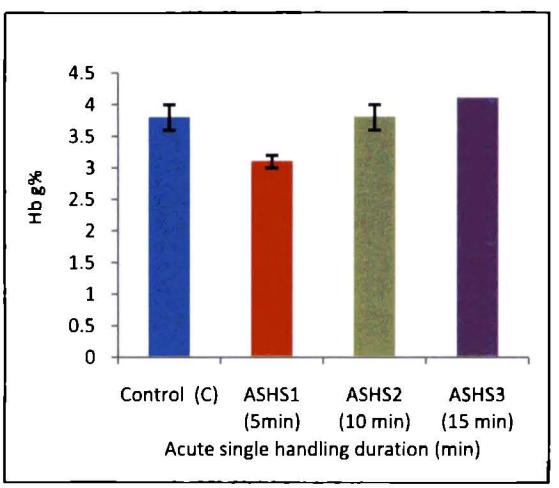


Figure 5

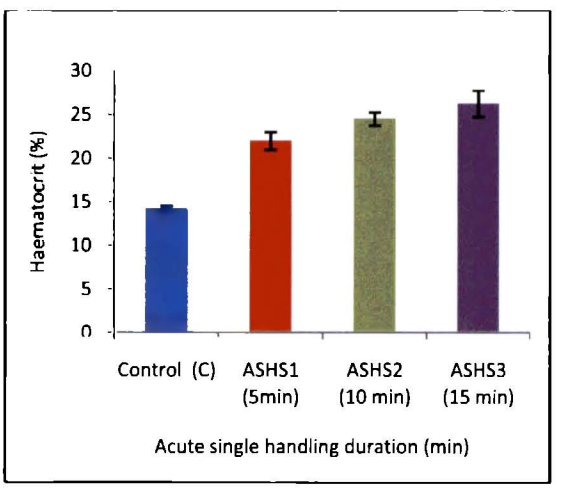


Figure 6

Figure 3. Variation in total erythrocyte count (TEC) (nox10^6/ml) of *Labeo rohita* subjected to acute single handling stress

Figure 4. Variation in total leukocyte count (TLC) (nox10^3/ml) of *Labeo rohita* subjected to acute single handling stress

Figure 5. Variation in haemoglobin (g/dl) of *Labeo rohita* subjected to acute single handling stress

Figure 6. Variation in haematocrit (%) of *Labeo rohita* subjected to acute single handling stress

4.1.2. Biochemical parameters

4.1.2.1. Glucose

The mean glucose level is represented in Table 5 and Figure 7. The mean glucose level in the unstressed fish (control) was significantly lower (30.00±1.00 mg/dl) and increased due to handling procedure. The glucose levels were found to be 45.00±1.00 mg/dl, 61.00±1.00 mg/dl, 74.50±0.00 mg/dl when fish were subjected to 5 min (ASHS1), 10 min (ASHS2) and 15 min (ASHS3) acute single handling stress, respectively.

4.1.2.2. Total protein

The mean total protein level in the unstressed fish was significantly higher (2.545±0.040 g/dl) which, however, decreased with the duration of handling stress (Figure 8). Mean protein contents were 2.39±0.00 g/dl in fish subjected to 5 min acute single handling stress (ASHS1), 2.45±0.05 g/dl in case of 10 min (ASHS2) and 2.23±0.11 mg /dl in the case of 15 min (ASHS3) (Table 5; Figure 8).

4.1.2.3. Triglycerides

Table 5 and Figure 9 illustrate variation in mean triglycerides in rohu subjected to acute single handling stress. The mean triglyceride content in the unstressed (control) fish was 24.00±2.00 mg/dl. It increased with the duration of handling stress. Maximum triglyceride (46.50±1.00 mg/dl) was recorded in ASHS3 (fish subjected to 15 min acute single handling stress) where as ASHS1 (fish subjected for 5 min acute single handling stress) had 41.00±0.00 mg/dl and an intermediate value of 43.00±1.00 mg/dl was noticed in ASHS2 (fish subjected to 10 min acute single handling stress).

4.1.2.4. Urea

The mean urea concentration in the unstressed (control) fish was 3.95±0.5 mg/dl which, however, increased with the duration of handling stress. Mean urea contents were 5.60±0.60 mg /dl in fish subjected to 5 min acute single handling stress (ASHS1), 6.60±0.60 mg/dl in case of 10 min (ASHS2) and 7.00±1.00 mg /dl in the case of 15 min (ASHS3) (Table 5; Figure 10).

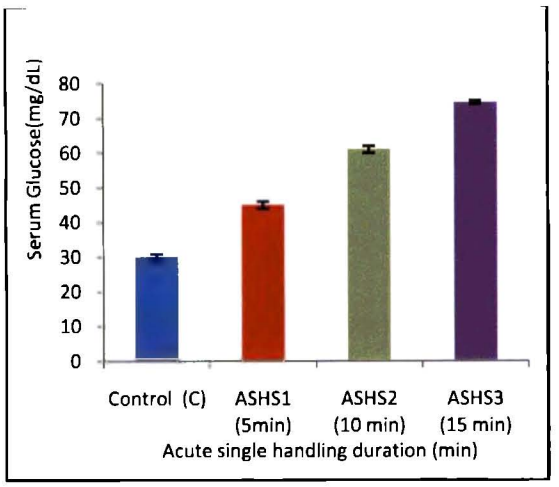
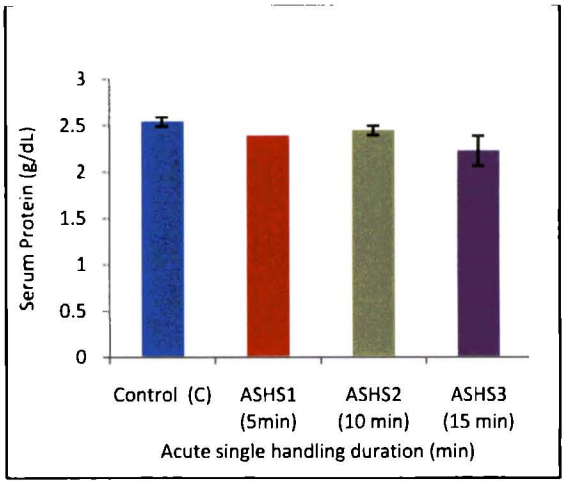


Figure 7



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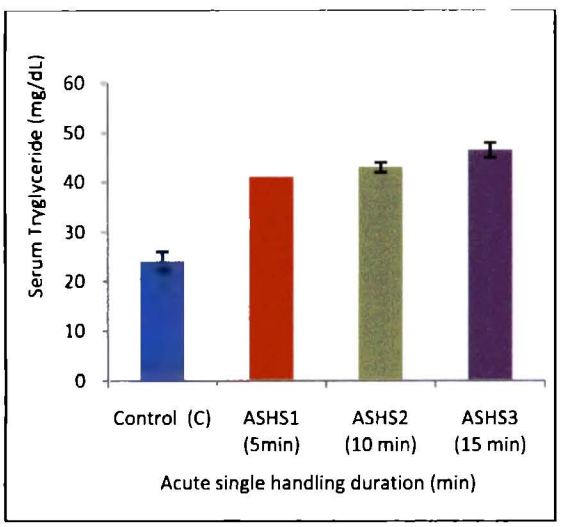


Figure 9

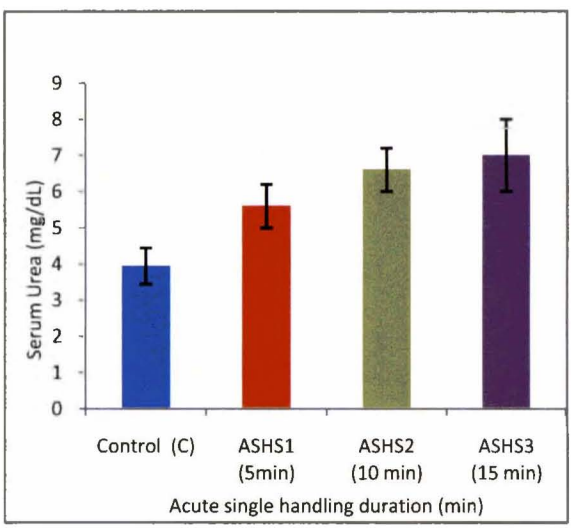


Figure 10

Figure 7. Variation in serum glucose (mg/dl) of *Labeo rohita* subjected to acute single handling stress

Figure 8. Variation in serum total protein (g/dl) of *Labeo rohita* subjected to acute single handling stress

Figure 9. Variation in serum triglyceride (mg/dl) of *Labeo rohita* subjected to acute single handling stress

Figure 10. Variation in serum urea (mg/dl) of *Labeo rohita* subjected to acute single handling stress

4.1.2.5. Creatinine

Fluctuation in mean creatinine content is represented in Table 5 and Figure 11. The mean creatinine in the unstressed (control) fish was 0.36±0.03 mg/dl. Maximum creatinine (0.40±0.02 mg/dl) was recorded in ASHS3 (fish subjected to 15 min acute single handling stress) where as ASHS1 (fish subjected for 5 min acute single handling stress) had 0.35±0.01 mg /dl and an intermediate value of 0.39±0.00 mg/dl was noticed in ASHS2 (fish subjected to 10 min acute single handling stress).

4.1.2.6. Alanine aminotransferase (ALT)

In the present study, the minimum ALT level (10.00±1.00 IU/L) was found in the unstressed rohu (control), and it showed an increased trend with acute and chronic handling duration (Table 5; Figure 12). The mean ALT values in rohu subjected to 5 min (ASHS1), 10 min (ASHS2) and 15 min (ASHS3) acute single handling stress were found to be 28.00±2.00 IU/L, 28.00±2.00 IU/L and 36.00±3.00 IU/L, respectively.

4.1.2.7. Aspartate aminotransferase (AST)

Table 5 and Figure 13 depict variation in AST levels during the present investigation. The lowest level of AST (41.00±2.00 IU/L) was found in the unstressed rohu (control), and it showed an increased trend with acute and chronic handling duration. The mean AST values in rohu subjected to 5 min (ASHS1), 10 min (ASHS2) and 15 min (ASHS3) acute single handling stress were found to be 56.00±3.50 IU/L, 58.00±2.00 IU/L and 60.00±3.00 IU/L, respectively.

4.1.2.8. Cortisol

Table 5 and Figure 14 illustrate variation in mean cortisol level observed in the present study. The mean cortisol level in the unstressed (control) fish was 20.00±2.00 µg/dl which, however, increased with the duration of handling stress. Mean cortisol levels were 30.00±1.00 µg/dl in fish subjected to 5 min acute single handling stress (ASHS1), 53.00±2.00 µg/dl in case of 10 min (ASHS2) and 56.00±0.00 µg /dl in the case of 15 min (ASHS3).

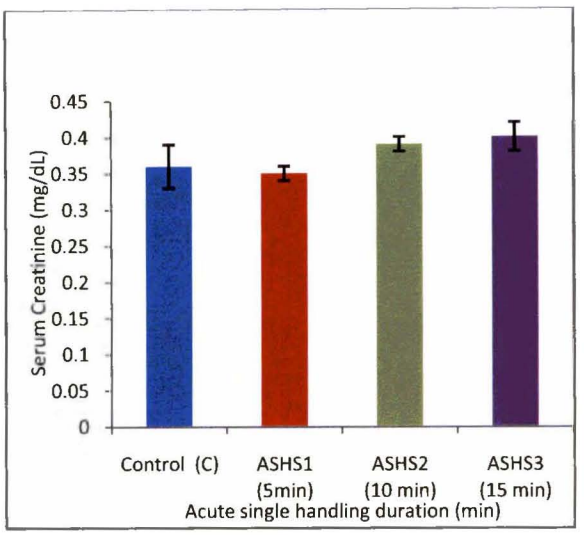


Figure 11

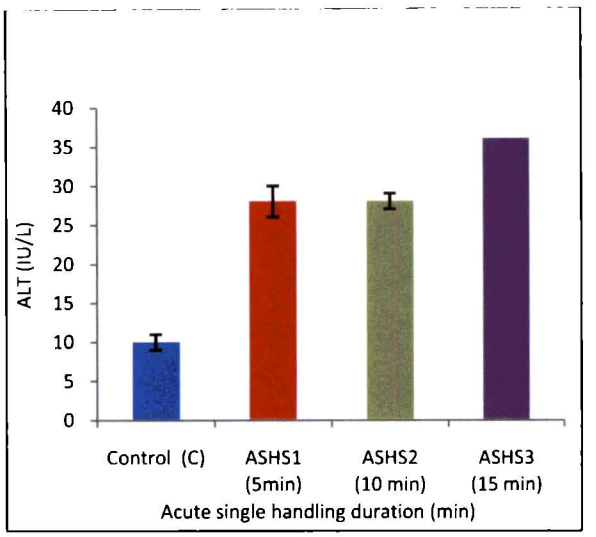


Figure 12

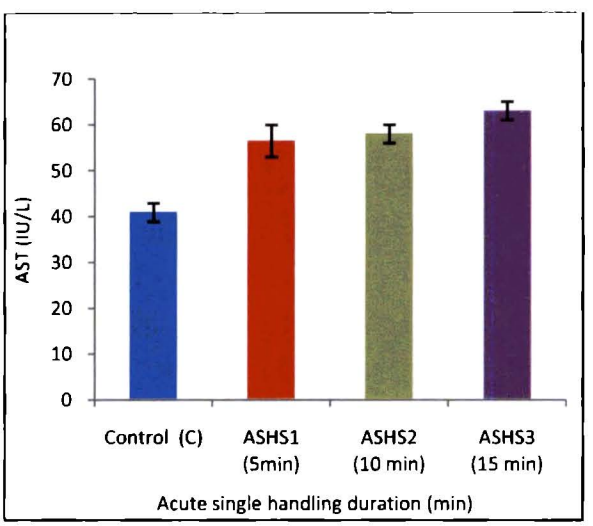


Figure 13

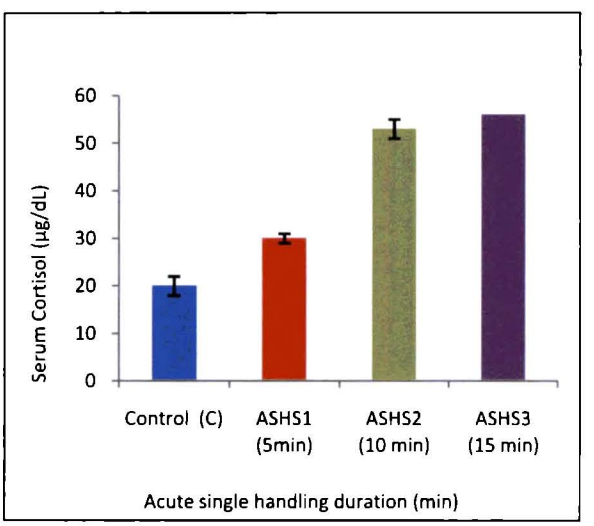


Figure 14

Figure 11. Variation in serum creatinine (mg/dl) of *Labeo rohita* subjected to acute single handling stress

Figure 12. Variation in serum alanine aminotransferase (IU/L) of *Labeo rohita* subjected to acute single handling stress

Figure 13. Variation in serum aspartate aminotransferase (IU/L) of *Labeo rohita* subjected to acute single handling stress

Figure 14. Variation in serum cortisol (µg/dl) of *Labeo rohita* subjected to acute single handling stress

4.2. Acute long term handling stress

Fluctuations in haematological and biochemical parameters of *Labeo rohita* subjected acute long term handling stress are presented in Table 6.

4.2.1. Haematological parameters

4.2.1.1. Total erythrocyte count (TEC) or Red blood corpuscles (RBC)

Table 6 and Figure 15 represent variations in TEC level. The mean TEC was found to be $1.17\pm0.01\times10^6$ no/ml in fish subjected to acute long term stress for 5 min (ALHS1) daily. It increased to $1.18\pm0.02\times10^6$ no/ml of blood when subjected for 10 min (ALHS2) and the highest value of $1.32\pm0.03\times10^6$ no/ml of blood was recorded when subjected for 15 min (ALHS3) (Table 6 and Figure 15).

4.2.1.2. Total leukocyte count (TLC) or White blood corpuscles (WBC)

The mean TLC values in rohu subjected to acute long term stress for 5 min/day (ALHS1), 10 min/day (ALHS2) and 15 min/day (ALHS3) were recorded as $52.00\pm1.00\times10^3$ no/ml, $56.00\pm2.00\times10^3$ no/ml and $59.00\pm3.00\times10^3$ no/ml, respectively (Table 6; Figure 16).

4.2.1.3. Haemoglobin

There was an increased trend in haemoglobin content of rohu subjected to acute long term handling stress. Fish subjected to 5 min acute long term handling stress (ALHS1) had mean haemoglobin level of 3.90±0.10 g/dl which increased to 4.20±0.10 g/dl and 4.25±0.00 g/dl in cases of fish subjected to 10 min (ALHS2) and 15 min (ALHS3) acute long term handling stress, respectively (Table 6; Figure 17).

4.2.1.4. Haematocrit

Mean haematocrit changes are illustrated in Table 6 and Figure 18. Mean haematocrit values recorded in the present study were found to be 11.09±037 %, 13.99±0.33 % and 16.95±0 % in rohu subjected to 5 min (ALHS1), 10 min (ALHS2), and 15 min (ALHS3) acute long term handling stress, respectively.

Table 6. Haematological and biochemical changes (mean ± standard deviation) in *Labeo rohita* subjected acute long term handling stress (ALHS) (n=6)

Parameter	Unstressed (Control) (C)	Stressed		
		ALHS1 (5 min/day)	ALHS2 (10 min/day)	ALHS3 (15 min/day)
Total erythrocyte count (TEC) or Red blood corpuscles (RBC) ($x10^6$ no/ml)	1.15 ± 0.05[a]	1.17 ±0.01[a]	1.18 ±0.02[a]	1.32 ±0.03[b]
Total leukocytes count (TLC) or White blood corpuscles (WBC) ($x10^3$ no/ml)	51.50 ± 0.50[a]	52.00 ±1.00[a]	56.00 ±2.00[a]	59.00 ±3.00[a]
Haemogiobln (Hb) (g/dl)	3.80 ±0.2.00[a]	3.90 ±0.1[a]	4.20 ±0.10[a]	4.25 ±0.05[a]
Haematocrit (Ht) (%)	14.20 ± 0.30[a]	11.09 ±0.37[b]	13.99 ±0.33[ab]	16.95 ±0.05[c]
Serum glucose (mg/dl)	30.00 ±1.00[a]	57.00 ±2.00[b]	83.50 ±0.50[c]	113.50 ±1.50[d]
Serum protein (g/dl)	2.54 ±0.05[a]	2.10 ±0.10[ab]	1.90 ±0.25[ab]	1.81 ±0.07[b]
Serum triglycerides (mg/dl)	24.00 ±2.00[a]	54.00 ±2.00[bd]	57.00 ±4.00[bd]	74.50 ±1.50[c]
Serum urea (mg/dl)	3.95 ±0.50[a]	1.75 ±0.03[b]	2.95 ±0.05[a]	3.45 ±0.35[a]
Serum creatinine (mg/dl)	0.36 ±0.03[a]	0.53 ±0.02[b]	0.64 ±0.02[bc]	0.74 ±0.03[bc]
Serum alanine aminotransferase (IU/L)	10 .00±1.00[a]	14.00 ±1.00[a]	33.00±0.00[b]	33.00 ±3.00[b]
Serum aspartate aminotransferase (IU/L)	41.00 ±2.00[a]	44.00 ±1.00[a]	52.50 ±0.50[b]	53.00 ±0.00[b]
Serum cortisol (µg/dl)	20.00 ±2.00[a]	26 .00±1.00[a]	29.00 ±1.00[a]	60.00 ±2.00[b]

***The values with different superscript differ significantly (P<0.05)**

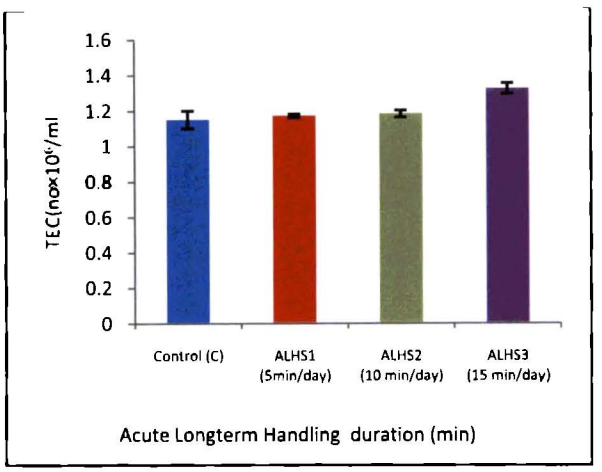


Figure 15

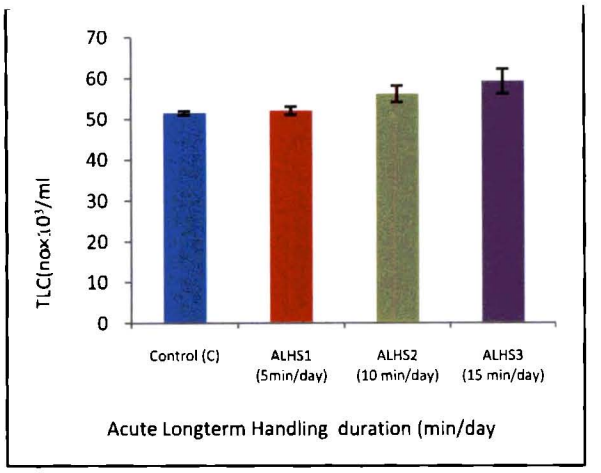
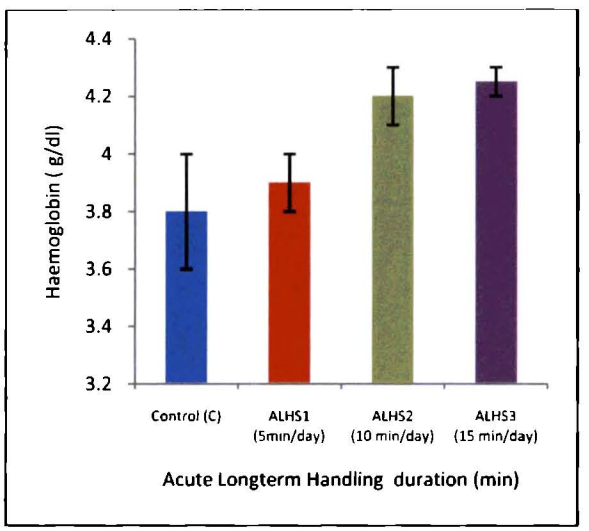


Figure 16



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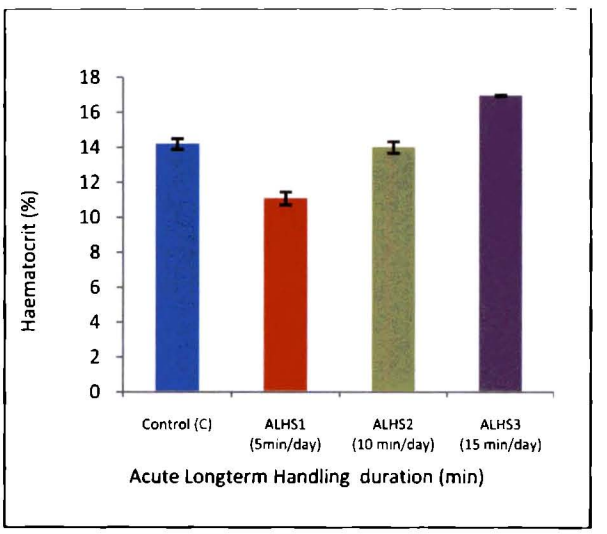


Figure 18

Figure 15. Variation in total erythrocyte count (TEC) (nox10^6/ml) of *Labeo rohita* subjected to acute long term (7 days) handling stress

Figure 16. Variation in total leukocyte count (TLC) (nox10^3/ml) of *Labeo rohita* subjected to acute long term (7 days) handling stress

Figure 17. Variation in haemoglobin (g/dl) of *Labeo rohita* subjected to acute long term (7 days) handling stress

Figure 18. Variation in haematocrit (%) of *Labeo rohita* subjected to acute long term (7 days) handling stress.

4.2.2. Biochemical parameters

4.2.2.1. Glucose

The mean glucose level was found to be 57.00±2.00 mg/dl, 83.56±0.50 mg/dl and 113.50±1.00 mg/dl in fish subjected to acute long term stress for 5 min (ALHS1), 10 min (ALHS2) and 15 min (ALHS3) daily, respectively (Table 6; Figure 19).

4.2.2.2. Total protein

Table 6 and Figure 20 portray fluctuations in protein content of rohu subjected to acute long term handling stress. Minimum protein content (1.82±0.07 g/dl) was observed in fish subjected to 15 min acute long term handling stress (ALHS3). Whereas the values recorded in 5 min handling stress (ALHS1) and 10 min handling stress (ALHS2) were found to be 2.10±0.10 g/dl and 1.91±0.26 g/dl, respectively.

4.2.2.4. Triglycerides

Mean triglyceride values recorded in 5 min acute long term handling stress (ALHS1), 10 min acute long term handling stress (ALHS2) and 15 min acute long term stress (ALHS3) were found to be 54.00±2.00 mg/dl, 57.00±4.00 mg/dl and 74.50±1.50 mg/dl, respectively (Table 6; Figure 21).

4.2.2.4. Urea

Variations in urea content in rohu subjected to acute long term handling stress are represented in Table 6 and Figure 22. Maximum urea content (6.45±2.00 mg/dl) was observed in fish subjected to 15 min acute long term handling stress (ALHS3). Whereas the values recorded in 5 min handling stress (ALHS1) and 10 min handling stress (ALHS2) were found to be 4.75±0.00 mg/dl and 5.95±0.05 mg/dl, respectively.

4.2.2.5. Creatinine

There was an increased trend in creatinine of rohu subjected to acute long term handling stress (Table 6; Figure 23). Fish subjected to 5 min acute long term handling stress (ALHS1) had mean creatinine level of 0.535±0.025 mg/dl which increased to 0.645±0.025 mg/dl and 0.745±0.03 mg/dl in cases of fish subjected to 10 min (ALHS2) and 15 min (ALHS3) acute long term handling stress, respectively.

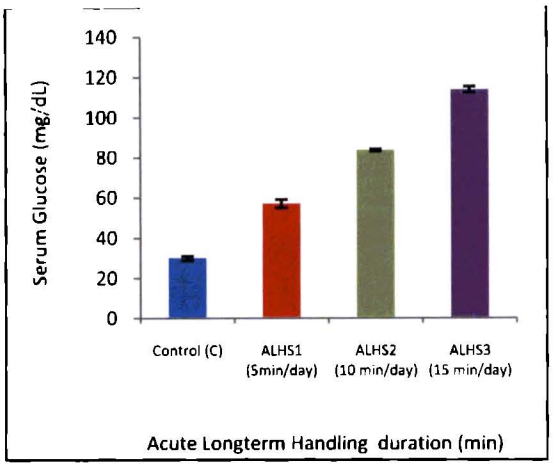
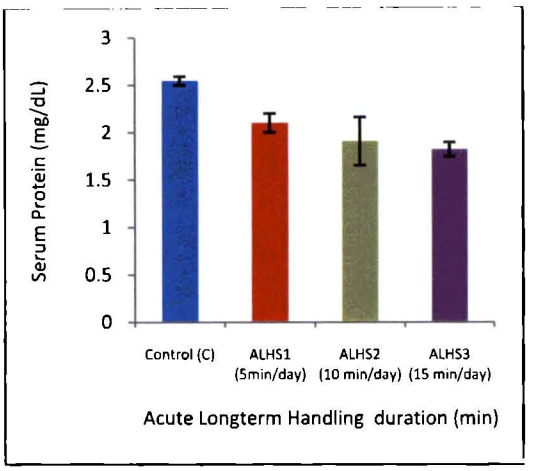


Figure 19



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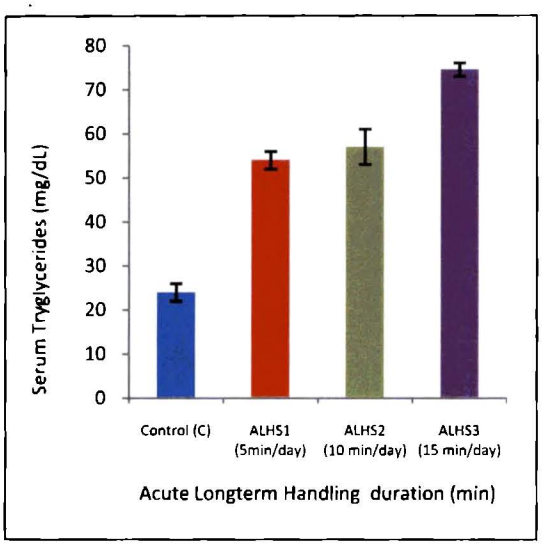

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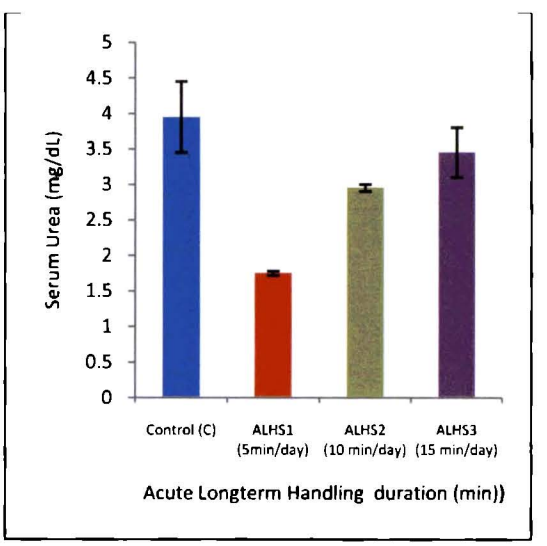

Figure 22

Figure 19. Variation in serum glucose (mg/dl) of *Labeo rohita* subjected to acute long term (7 days) handling stress

Figure 20. Variation in serum total protein (g/dl) of *Labeo rohita* subjected to acute long term (7 days) handling stress

Figure 21. Variation in serum triglyceride (mg/dl) of *Labeo rohita* subjected to acute long term (7 days) handling stress

Figure 22. Variation in serum urea (mg/dl) of *Labeo rohita* subjected to acute long term (7 days) handling stres

4.2.2.6. Alanine aminotransferase (ALT)

The mean ALT values in rohu subjected to acute long term stress for 5 min/day (ALHS1), 10 min/day (ALHS2) and 15 min/day (ALHS3) were recorded as 14.00±1.00 IU/L, 33.00±0.00 IU/L and 33.30±0.00 IU/L, respectively (Table 6; Figure 24).

4.2.2.7. Aspartate aminotransferase (AST)

The mean AST values in rohu subjected to acute long term stress for 5 min/day (ALHS1), 10 min/day (ALHS2) and 15 min/day (ALHS3) were recorded as 44.00±1.00 IU/L, 52.50±0.50 IU/L and 53.00±0.00 IU/L, respectively (Table 6; Figure 25).

4.2.2.8. Cortisol

Table 6 and Figure 26 depict mean cortisol variations in rohu subjected to acute long term handling stress. Maximum cortisol content (60.00±2.00 µg/dl) was observed in fish subjected to 15 min acute long term handling stress (ALHS3). Whereas the values recorded in 5 min handling stress (ALHS1) and 10 min handling stress (ALHS2) were found to be 26.00±1.00 µg/dl and 29.00±1.00 µg/dl, respectively.

4.3. Transportation stress

Variations in haematological and biochemical parameters of *Labeo rohita* subjected transportation stress are presented in Table 7.

4.3.1. Haematological parameters

4.3.1.1. Haemoglobin

Haemoglobin content showed significant variation with transportation stress as well as density of packing in the present investigation (Table 7; Figure 27). The mean Hb value in fish of BD group (before disturbance – control) was 3.80±0.02 g/dl and it increased to 4.05±0.15 g/dl before transportation (BT). The mean Hb values further increased to 5.40±0.00 g/dl in 500 g/bag packing density (AT1), 4.70±0.02 g/dl in 1.0 kg/bag packing density (AT2) and 5.20±0.02 g/dl in 1.5 kg/bag packing density (AT3) immediately after transportation .

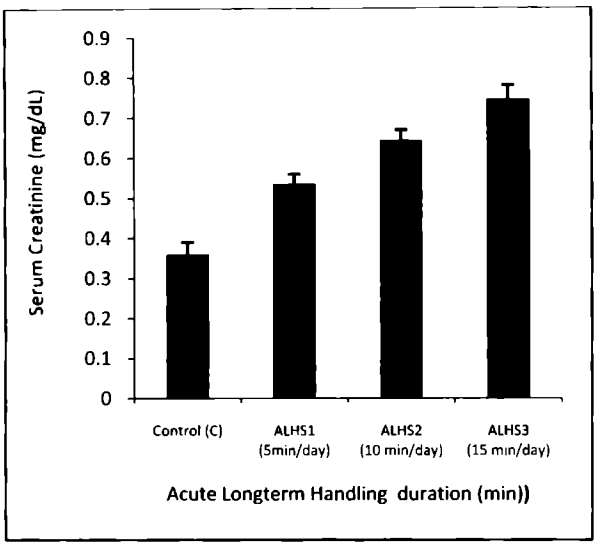

Figure 23

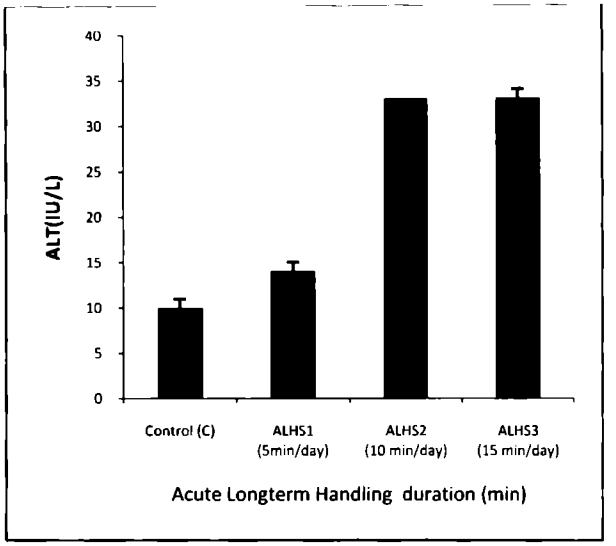

Figure 24

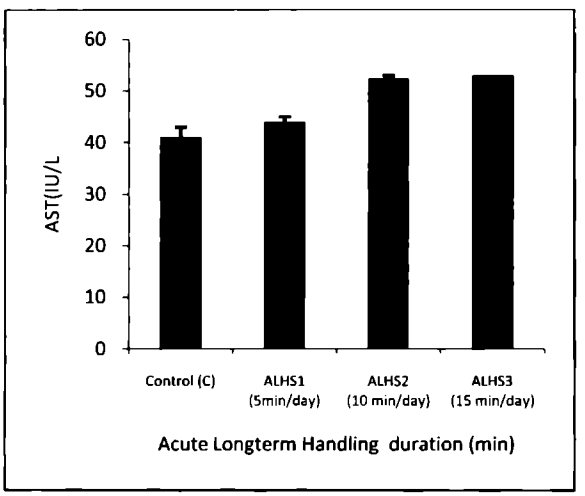

Figure 25

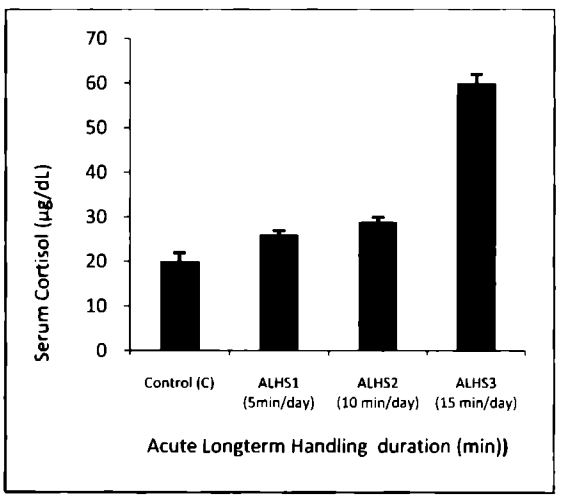

Figure 26

Figure 23. Variation in serum creatinine (mg/dl) of *Labeo rohita* subjected to acute long term (7 days) handling stress

Figure 24. Variation in serum alanine aminotransferase (IU/L) of *Labeo rohita* subjected to acute long term (7 days) handling stress

Figure 25. Variation in serum aspartate aminotransferase (IU/L) of *Labeo rohita* subjected to acute long term (7 days) handling stress

Figure 26. Variation in serum cortisol (mg/dl) of *Labeo rohita* subjected to acute long term (7 days) handling stress.

Table 7. Haematological and biochemical changes (mean ± standard deviation) in *Labeo rohita* during transportation procedure at different packing densities (n=15)

***The values with different superscript differ significantly (P<0.05)**

Parameters	Before disturbance (BD)	Before transportation (BT)	After transportation (AT)		
			AT1 (500 g/bag)	AT2 (1 kg/bag)	AT3 1.5 kg/bag
Haemoglobin (Hb) (g/dl)	3.80 ±0.20^{a}	4.05 ±0.15^{a}	5.40 ±0.00^{b}	4.70 ±0.20^{a}	5.20 ±0.20^{b}
Haematocrit (Ht) (%)	14.20±0.30^{a}	18.93 ±0.28^{a}	27.82 ±0.82^{b}	34.00 ±0.96^{c}	36.93 ±1.93^{cd}
Serum glucose (mg/dl)	30.00 ±1.00^{a}	41.00 ±3.00^{b}	52.50 ±3.50^{b}	61.00 ±2.00^{cd}	62.00 ±1.00^{d}
Serum protein (g/dl)	8.28 ±0.28^{a}	7.53 ±0.27^{a}	4.42 ±0.21^{b}	2.00 ±0.37^{c}	3.81 ±0.01^{d}
Serum tryglycerides (mg/dl)	24.00 ±2.00^{a}	39 .00±3.00^{ab}	54.00 ±2.00^{b}	57.00 ±1.50^{b}	81.50 ±1.50c
Serum creatinine (mg/dl)	0.36 ±0.03^{a}	0.35 ±0.01^{a}	0.45 ±0.04^{a}	0.59 ±0.03^{bc}	0.65 ±0.02^{bc}
Serum alanine aminotransferase (IU/L)	10.00 ±1.00^{a}	16.00 ±0.00^{b}	18 .00±2.00^{b}	24.00 ±4.00^{bc}	32.00 ±2.00^{c}
Serum aspartate aminotransferase (IU/L)	24.00 ±2.00^{a}	25.00 ±1.00^{a}	28.00 ±0.00^{a}	29.00 ±2.00^{ab}	35.00 ±0.50^{b}
Serum lactate dehydrogenase (IU/L)	71.00 ±6.00^{a}	133.00 ±3.00^{b}	182.00 ±2.00^{cd}	132.00 ±2.00^{b}	192.00 ±2.00^{d}
Serum cortisol (µg/dl)	20.00 ±2.00^{a}	29.00 ±3.00^{a}	52.00 ±2.00^{b}	55.00 ±1.00^{b}	56.00 ±0.00^{b}

4.3.1.2. Haematocrit

The present investigation implied a significant variation in haematocrit value with transportation procedures (Table 7; Figure 28). Mean haematocrit in the undisturbed fish (BD) were significantly lower (14.20±0.30 %) and increased as the transportation procedure begun. Before transportation (BT) it was 18.93±0.28 %, and increased to 27.82±0.82 %, 34.96±0.96 %, and 36.93±1.93 % in the fish packed at the rate of 500 g/bag (AT1), 1 kg /bag (AT2) and 1.5 kg/bag (AT3).

4.3.2. Biochemical parameters

4.3.2.1. Glucose

Glucose level varied with transportation stress as well as density of packing in the present research. The mean glucose level in fish of BD group (before disturbance – control) was 30.00±2.00 mg/dl and it increased to 41.00±3.00 mg/dl before transportation (BT). The mean glucose values further increased to 52.50±3.50 mg/dl in 500 g/bag packing density (AT1), 61.00±2.00 mg/dl in 1.0 kg/bag packing density (AT2) and 62.00±1.00 mg/dl in 1.5 kg/bag packing density (AT3) immediately after transportation (Table 7; Figure 29).

4.3.2.2. Total protein

The serum protein altered with transportation procedures as well as density of packing in comparison to undisturbed fishes. The mean total protein level in the unstressed fish (BD) was relatively higher (8.28±0.28 g/dl) and decreased with the stress. It decreased as the transportation procedure proceeded. The value was 7.53±0.27 g/dl before transportation (BT). Immediately after transportation it further declined to 4.42±0.21 g/dl, 2.00±0.37 g/dl, and 3.81±0.10 g/dl in fish stocked at 500 g/bag (AT1), 1 kg /bag (AT2) and 1.5 kg/bag (AT3), respectively (Table 7; Figure 30).

4.3.2.3. Triglycerides

The serum triglycerides showed variation with transportation procedures as well as density of packing in comparison to undisturbed fishes. The mean triglcerides in the unstressed fish (BD) was relatively lower (24.00±2.00 mg/dl) and it increased with the stress. The value increased to 39.00±3.00 mg/dl before transportation (BT). Immediately after transportation it further increased to 54.00±2.00 mg/dl, 58.50±1.50 mg/dl, and 81.50±1.50 mg/dl in fish stocked at 500 g/bag (AT1), 1 kg /bag (AT2) and 1.5 kg/bag (AT3), respectively (Table 7; Figure 31) .

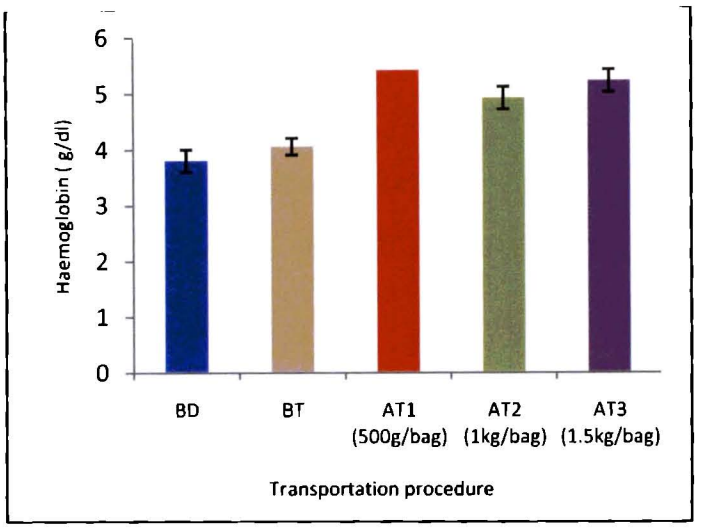

Figure 27

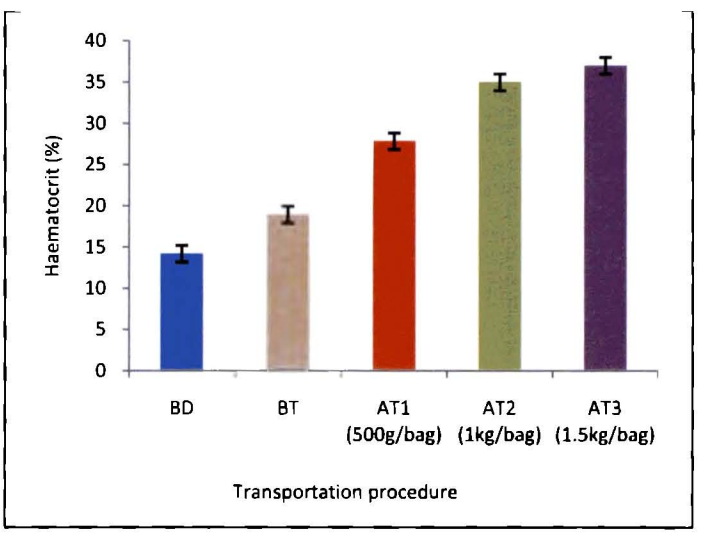

Figure 28

Figure 27. Variation in haemoglobin (g/dl) of *Labeo rohita* during transportation procedure

Figure 28. Variation in haematocrit (%) of *Labeo rohita* during transportation procedure

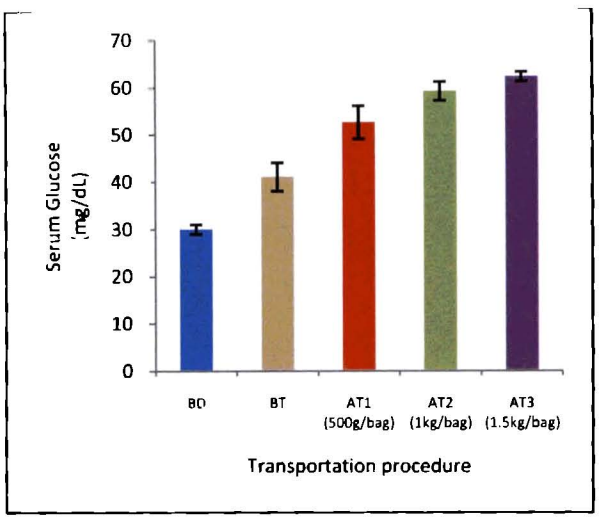

Figure 29

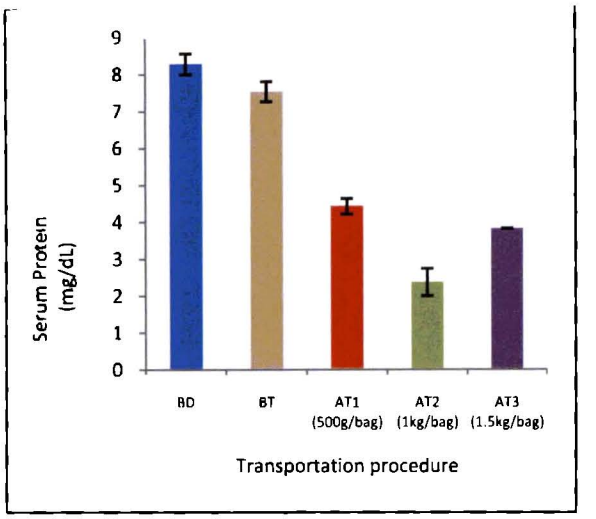

Figure 30

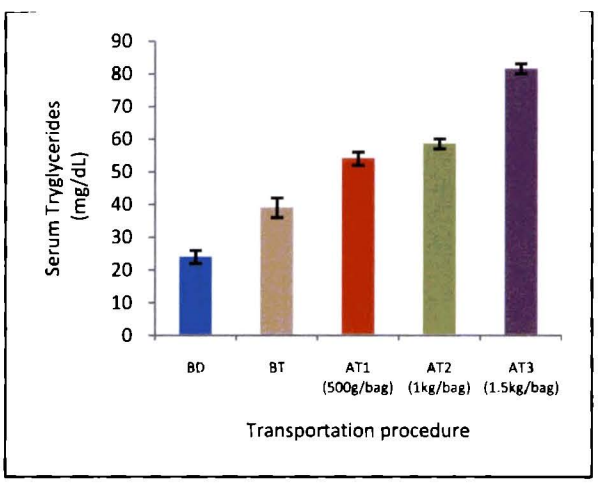

Figure 31

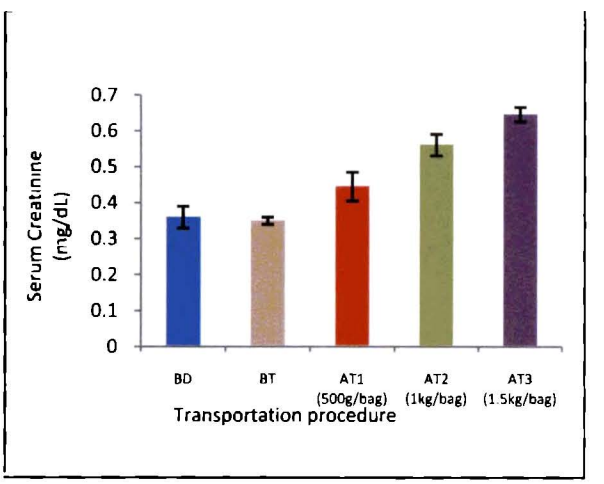

Figure 32

Figure 29. Variation in serum glucose (mg/dl) of *Labeo rohita* during transportation procedure

Figure 30. Variation in serum total protein (g/dl) of *Labeo rohita* during transportation procedure

Figure 31. Variation in serum triglyceride (mg/dl) of *Labeo rohita* during transportation procedure

Figure 32. Variation in serum creatinine (mg/dl) of *Labeo rohita* during transportation procedure

4.3.2.4. Creatinine

Variations in creatinine level in rohu subjected to transportation stress in Table 7 and Figure 32. The mean creatinine level in fish of BD group (before disturbance – control) was 0.350±0.030 mg/dl and it decreased to 0.350±0.000 mg/dl before transportation (BT). The mean creatinine values further increased to 0.445±0.035 mg/dl in 500 g/bag packing density (AT1), 0.590±0.030 mg/dl in 1.0 kg/bag packing density (AT2) and 0.650±0.030 mg/dl in 1.5 kg/bag packing density (AT3) immediately after transportation.

4.3.2.5. Alanine aminotransferase (ALT)

The serum ALT level varied with transportation stress as well as density of packing in the present research and the results obtained are presented in Table 7 and Figure 33. The mean ALT level in fish of BD group (before disturbance – control) was 10.00±1.00 IU/L and it increased to 16.00±0.00 IU/L before transportation (BT). The mean ALT values further increased to 18.00±2.00 IU/L in 500 g/bag packing density (AT1), 24.00±4.00 IU/L in 1.0 kg/bag packing density (AT2) and 32.00±2.00 IU/L in 1.5 kg/bag packing density (AT3) immediately after transportation.

4.3.2.6. Aspartate aminotransferase (AST)

The present investigation also implied a significant variation in AST levels with transportation procedures. Mean AST in the undisturbed fish (BD) was low (24.00±2.00 IU/L) and increased as the transportation procedure begun. Before transportation (BT) it was 25.00±1.00 IU/L, and increased to 28.00±0.00 IU/L, 29.00±2.00 IU/L and 35.00±1.00 IU/L in the fish packed at the rate of 500 g/bag (AT1), 1 kg /bag (AT2) and 1.5 kg/bag (AT3), respectively (Table 7; Figure 34).

4.3.2.7. Lactate dehudrogenase (LDH)

The present study revealed a variation in mean LDH level with transportation stress as well as density of packing. The mean LDH level in fish of BD group (before disturbance – control) was 71.00±6.00 IU/L and it increased to 133.00±3.00 IU/L before transportation (BT). The mean glucose values recorded were 133.00±3.00 IU/L in 500 g/bag packing density (AT1), 134.00±2.00 IU/L in 1.0 kg/bag packing density (AT2) and the highest value of 192.00±2.00 IU/L in 1.5 kg/bag packing density (AT3) immediately after transportation (Table 7; Figure 35).

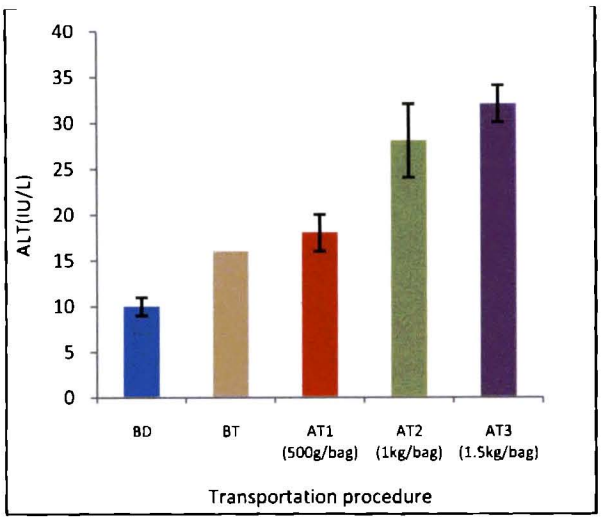

Figure 33

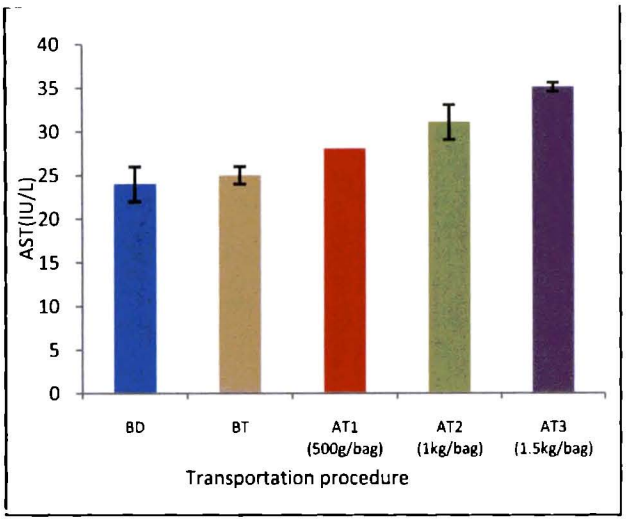

Figure 34

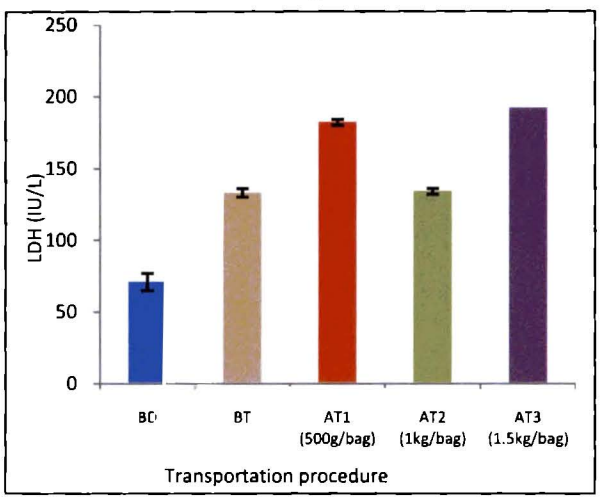

Figure 35

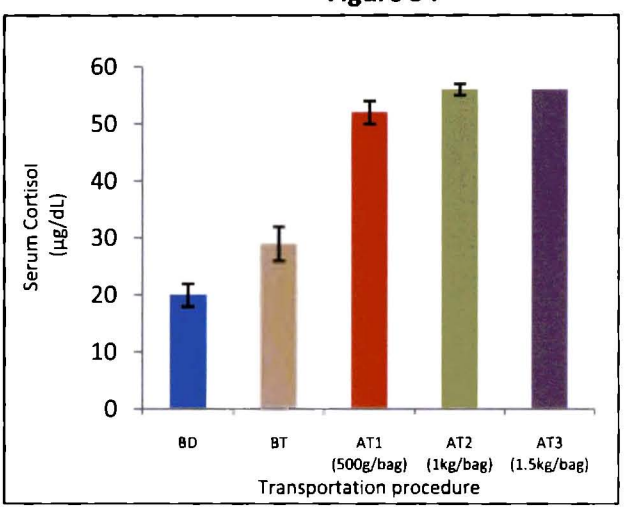

Figure 36

Figure 33. Variation in serum alanine aminotransferase (IU/L) of *Labeo rohita* during transportation procedure

Figure 34. Variation in serum aspartate aminotransferase (IU/L) of *Labeo rohita* during transportation procedure

Figure 35. Variation in lactate dehydrogenase (IU/L) of *Labeo rohita* during transportation procedure

Figure 36. Variation in serum cortisol (µg/dl) of *Labeo rohita* during transportation procedure

4.3.2.8. Cortisol

The changes in serum cortisol during transportation procedure are depicted in Table 7 and Figure 36. The mean cortisol level in the unstressed fish (BD) was relatively lower (20.00±2.00 µg/dl) and increased as the transportation procedure begun. The value was 29.00±3.00 µg/dl before transportation (BT). Immediately after transportation it further augmented to 52.00±2.00 µg/dl, 55.00±1.00 µg/dl, and 56.00±0.00 µg/dl in fish stocked at 500 g/bag (AT1), 1 kg /bag (AT2) and 1.5 kg/bag (AT3), respectively.

4.4. Bacteriological stress

Alterations in haematological and biochemical parameters of *Labeo rohita* subjected bacteriological stress are presented in Table 8.

4.4.1. Haematological parameters

4.4.1.1. Total erythrocyte count (TEC) or Red blood corpuscles (RBC)

In the present investigation the mean TEC value of control (initial) rohu was $1.26\pm0.03\times10^6$ no/ml of blood. After 6 hrs the TEC increased to $1.40\pm0.04\times10^6$ no/ml of blood and after 7 days it further increased to $1.45\pm0.01\times10^6$ no/ml of blood when subjected to *Aeromonas hydrophila* inoculation at sublethal dose (Table 8; Figure 37). Likewise the TEC values in rohu increased to $1.32\pm0.01\times10^6$ no/ml of blood and $1.42\pm0.00\times10^6$ no/ml of blood when subjected to *Edwardsiella tarda* inoculation at sublethal dose for 6 hours and 7 days, respectively (Table 8; Figure 37).

4.4.1.2. Total leukocyte count (TLC) or White blood corpuscles (WBC)

The mean TLC value of rohu in control (initial) was $44.00\pm2.00\times10^3$ no/ml of blood. After 6 hrs it increased to $64.00\pm3.00\times10^3$ no/ml of blood, however, after 7 days it decreased to $46.50\pm0.50\times10^3$ no/ml of blood when subjected to *Aeromonas hydrophila* inoculation at sublethal dose (Table 8; Figure 38). The mean TLC values obtained were $49.50\pm0.50\times10^3$ no/ml of blood and $41.20\pm1.50\times10^3$ no/ml of blood after 6 hours and 7 days, respectively, when rohu was experimentally challenged with *Edwardsiella tarda* at sublethal dose (Table 8; Figure 38).

4.4.1.3. Haemoglobin

In the present study the haemoglobin level of control (initial) rohu was 4.00±0.20 g/dl of blood. After 6 hrs the value decreased to 3.80±0.00 g/dl and after 7 days it increased to 4.10±0.20 g/dl when subjected to *Aeromonas hydrophila* inoculation at sublethal dose. (Table 8; Figure 39). However, the haemoglobin concentration values in rohu initially increased to 4.10±0.20 g/dl after 6 hrs while decreased to 4.00±0.00 g/dl after seven days when experimentally infected with sublethal doses of *Edwardsiella tarda* (Table 8; Figure 39).

Table 8. Haematological and biochemical changes (mean ± standard deviation) in *Labeo rohita* challenged with sublethal doses of *Aeromonas hydrophila* and *Edwardsiella tarda (n=12)*

Parameter	Uninfected (Control) (C)	Infected			
		After 6 hrs		After 7 days	
		A. hydrophila	*E. tarda*	*A. hydrophila*	*E. tarda*
Total erythrocyte count (TEC) or Red blood corpuscles (RBC) ($x10^6$ no/ml)	1.26 ±0.03^{aA}	1.40 ±0.04^{ab}	1.32 ±0.01^{A}	1.45 ±0.01^{b}	1.42 ±0.00^{B}
Total leukocytes count (TLC) or White blood corpuscles (WBC) ($x10^3$ no/ml)	44.00 ±2.00^{aA}	64.00 ±3.00^{b}	49.50 ±0.50^{A}	46.50 ±1.50^{a}	41.50 ±1.50^{B}
Haematocrit (Ht) (%)	15.00 ±1.00^{aA}	24.80±1.80^{b}	18.22 ±0.22^{A}	24.40 ±2.40^{b}	23.42 ±2.42^{A}
Haemoglobin (Hb) (g/dl)	4 .00±0.20^{aA}	3.80 ±0..44^{a}	4.10 ±0.20^{A}	4.10 ±0.20^{a}	4.00 ±0.00^{A}
Serum glucose (mg/dl)	68.00 ±2.00^{aA}	73.00 ±1.00^{a}	70.00 ±1.00^{A}	71.50 ±1.50^{a}	70.00 ±0.01^{A}
Serum protein (g/dl)	3.18 ±0.18^{aA}	2.49 ±0.49^{a}	3.15.00 ±0.15^{aA}	2.89 ±0.06^{a}	3.31 ±0.02^{A}
Serum triglycerides (mg/dl)	54.00 ±2.00^{aA}	81.50 ±1.50^{b}	58.50 ±1.50^{A}	111.50 ±1.50^{c}	79.00 ±1.00^{B}
Serum urea (mg/dl)	3.95 ±0.50^{aA}	7.00 ±1.00^{a}	6.60±0.60^{A}	5.60±0.60^{a}	5.30±0.60^{A}
Serum creatinine (mg/dl)	0.28 ±0.02^{aA}	0.44 ±0.02^{b}	0.32 ±0.02^{A}	0.29 ±0.01^{b}	0.27 ±0.01^{A}
Serum alanine aminotransferase (IU/L)	21.00 ±1.00^{aA}	59.00 ±2.00^{b}	27.00 ±2.00^{A}	31.00 ±2.00^{c}	21.00 ±2.00^{A}
Serum aspartate aminotransferase (IU/L)	37.00 ±2.00^{aA}	67.00 ±2.00^{b}	37.00 ±2.00^{A}	34.00 ±2.00^{c}	36.00 ±2.00^{A}
Serum lactate dehydrogenase (IU/L)	109.00 ±2.00^{aA}	170.00 ±4.00^{b}	117.00 ±2.00^{A}	76.00 ±2.00^{b}	79.50 ±0.50^{B}
Serum cortisol (µg/dl)	19.00 ±1.00^{aA}	30.00 ±2.00^{b}	25.00 ±1.00^{A}	24.00 ±1.00^{b}	23.00 ±1.00^{A}

The values with different superscript differ significantly (P<0.05)

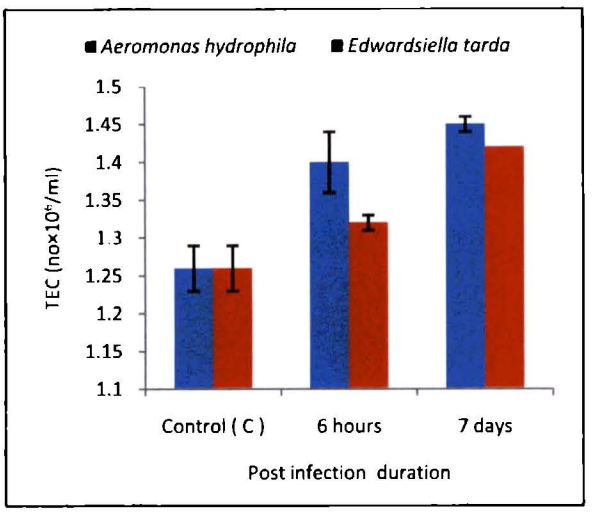

Figure 37

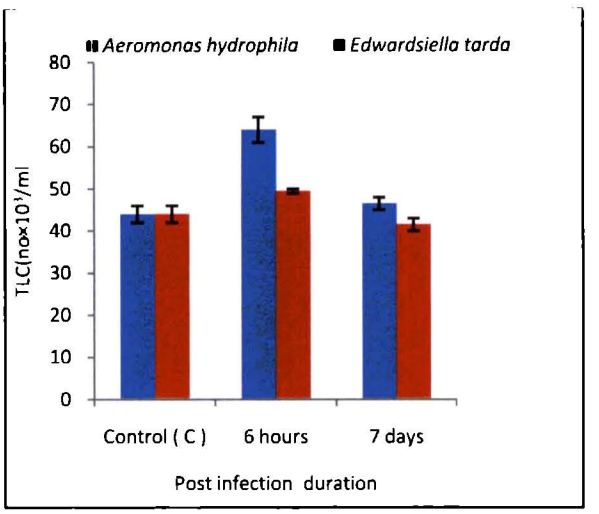

Figure 38

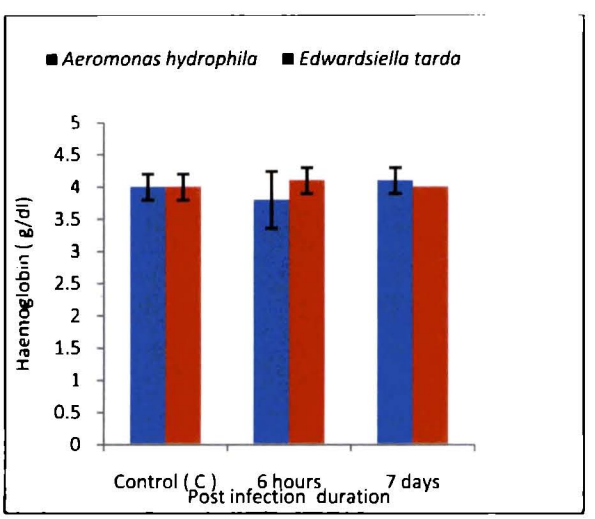

Figure 39

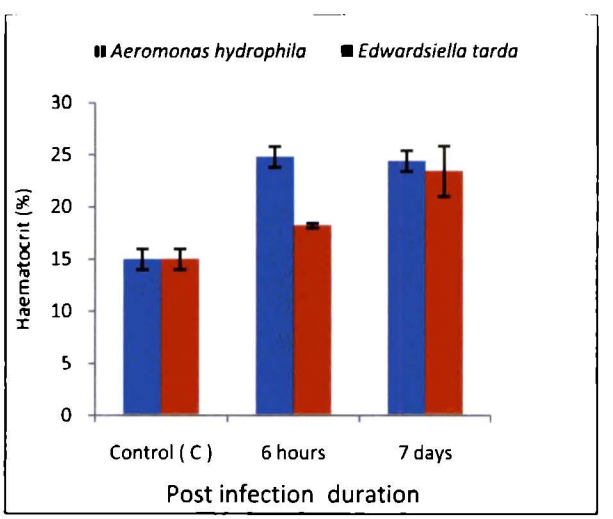

Figure 40

Figure 37. Variation in total erythrocyte count (TEC) (nox10^6/ml) of *Labeo rohita* challenged with sublethal doses of *Aeromonas hydrophila* and *Edwardsiella tarda*

Figure 38. Variation in total leukocyte count (TLC) (nox10^3/ml) of *Labeo rohita* challenged with sublethal doses of *Aeromonas hydrophila* and *Edwardsiella tarda*

Figure 39. Variation in haemoglobin (g/dl) of *Labeo rohita* challenged with sublethal doses of *Aeromonas hydrophila* and *Edwardsiella tarda*

Figure 40. Variation in haematocrit (%) of *Labeo rohita* subjected to challenged with sublethal doses of *Aeromonas hydrophila* and *Edwardsiella tarda*

4.4.1.4. Haematocrit

The mean haematocrit value of rohu in control (initial) was 15.00±1.00 %. After 6 hrs it increased to 24.80±1.80 %, however, after 7 days it decreased to 18.22±0.22 % when experimentally challenged with *Aeromonas hydrophila* inoculation at sublethal dose (Table 8; Figure 40). The mean haematocrit values obtained were 24.40±2.40 % and 23.42±2.42 % after 6 hours and 7 days, respectively, when rohu was experimentally challenged with *Edwardsiella tarda* at sublethal dose (Table 8; Figure 40).

4.4.2. Biochemical parameters

4.4.2.1. Glucose

The present study indicated that glucose level of unstressed (control) rohu which was 68.00±2.00 mg/dl increased to 73.00±1.00 mg/dl after 6 hrs and decreased to 71.50±1.50 mg/dl after seven days in experimentally infected with sublethal dose of *Aeromonas hydrophila* (Table 8; Figure 41). Similar trend was observed in *Edwardsiella tarda* infected (sublethal level) rohu in which glucose level rose to 70.00±1.00 mg/dl and remained at the same level even after seven days (Table 8; Figure 41).

4.4.2.2. Total protein

The mean protein content of rohu in control (initial) was 3.18±0.18 g/dl. After 6 hrs it decreased to 2.49±0.49 mg/dl, however, after 7 days it increased to 2.89±0.07 mg/dl when experimentally challenged with *Aeromonas hydrophila* infection at sublethal dose (Table 8; Figure 42). Likewise, the mean protein content obtained were 3.15±0.15 mg/dl and 3.31±0.05 mg/dl after 6 hours and 7 days, respectively, when rohu was experimentally challenged with *Edwardsiella tarda* at sublethal dose (Table 8; Figure 42).

4.4.2.3. Triglycerides

In present study the triglyceride level of uninfected fish was 54.00±2.00 mg/dl. But after 6 hrs it increased to 110.00±1.50 mg/dl and 79.00±1.50 mg/dl when it was challenged with sublethal doses of *Aeromonas hydrophila* and *Edwardsiella tarda*, respectively (Table 8; Figure 43). After 7 days it the corresponding values declined to 81.50±1.50 mg/dl and 58.5±1 mg/dl in *Aeromonas hydrophila* and *Edwardsiella tarda* infected fish.

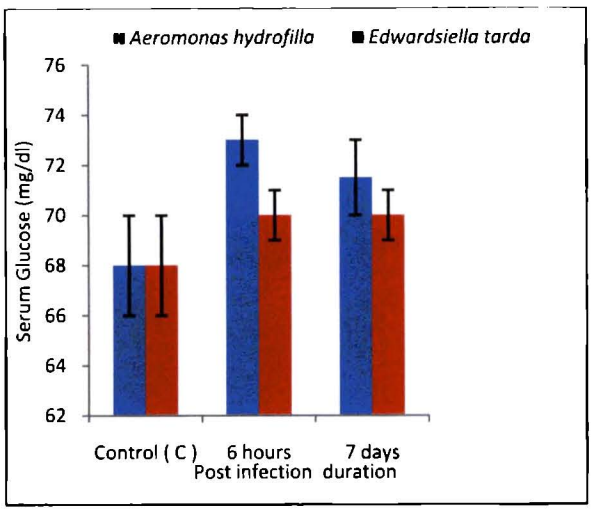

Figure 41

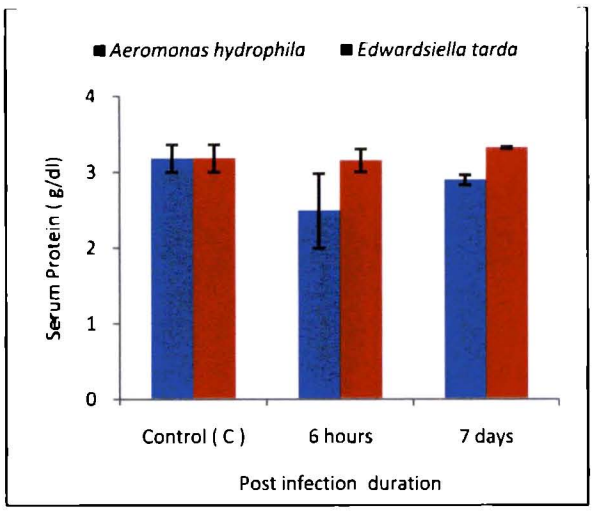

Figure 42

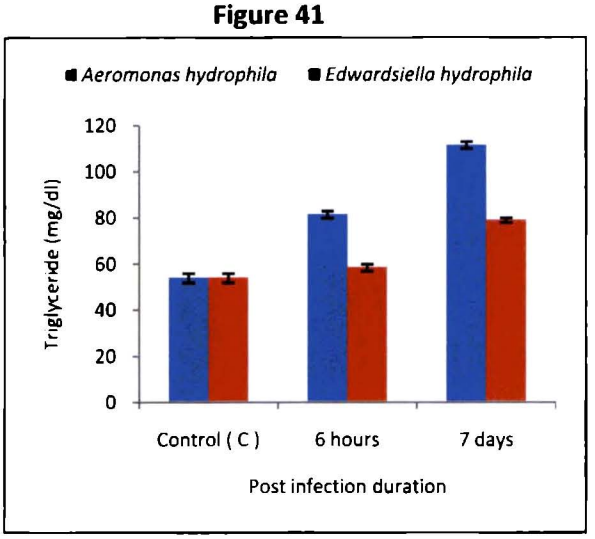

Figure 43

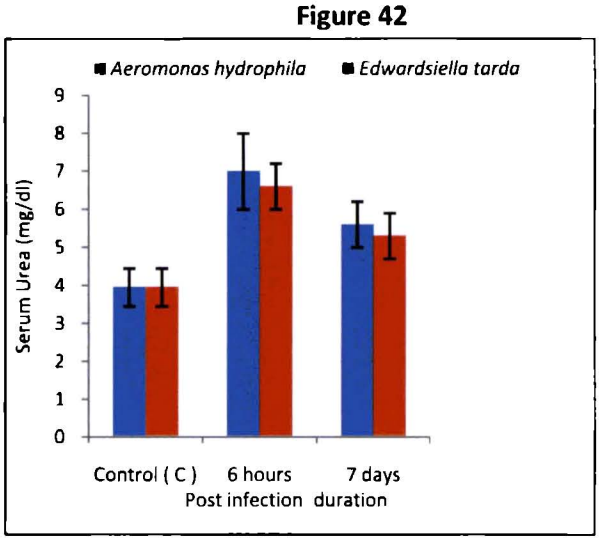

Figure 44

Figure 41. Variation in serum glucose (mg/dl) of *Labeo rohita* challenged with sublethal doses of *Aeromonas hydrophila* and *Edwardsiella tarda*

Figure 42. Variation in serum total protein (g/dl) of *Labeo rohita* challenged with sublethal doses of *Aeromonas hydrophila* and *Edwardsiella tarda*

Figure 43. Variation in serum triglyceride (mg/dl) of *Labeo rohita* challenged with sublethal doses of *Aeromonas hydrophila* and *Edwardsiella tarda*

Figure 44. Variation in serum urea (mg/dl) of *Labeo rohita* challenged with sublethal doses of *Aeromonas hydrophila* and *Edwardsiella tarda*

4.4.2.4. Urea

The mean urea content of rohu in control (initial) was 3.95±0.5 mg/dl. After 6 hrs it increased to 7±1 mg/dl, however, after 7 days it decreased to 5.6±0.6 mg/dl when experimentally challenged with *Aeromonas hydrophila* infection at sublethal dose (Table 8; Figure 44). Likewise, the mean urea level obtained were 6.6±0.6 mg/dl and 5.3±0.6 mg/dl after 6 hours and 7 days of post infection respectively, when rohu was experimentally challenged with *Edwardsiella tarda* at sublethal dose (Table 8; Figure 44).

4.4.2.5. Creatinine

In the present analysis uninfected rohu had creatinine level of 0.28±0.02 mg/dl. But after 6 hrs it increased to 0.44±0.02 mg/dl but again declined to 0.29±0.01 mg/dl when subjected to *Aeromonas hydrophila* inoculation at sublethal dose (Table 8; Figure 45). Similar trend was observed in fish experimentally challenged with *Edwardsiella tarda*, wherein corresponding values after 6 hrs and 7 days were found to be 0.32±0.02 mg/dl and 0.27±0.01 mg/dl, respectively (Table 8; Figure 45).

4.4.2.6. Alanine aminotransferase (ALT)

In the present study the mean ALT level of uninfected (control) fish was 21.00±1.00 IU/L. But after 6 hrs it increased to 59.00±2.00 IU/L, however, after 7 days it decreased to 31.00±2.00 IU/L in rohu experimentally challenged with *Aeromonas hydrophila* inoculation at sublethal dose (Table 8; Figure 46). Similarly, mean ALT level increased to 27.00±2.00 IU/L after 6 hrs and decreased to 21.00±2.00 IU/L after 7 days in rohu subjected to *Edwardsiella tarda* inoculation at sublethal dose (Table 8; Figure 46).

4.4.2.7. Aspartate aminotransferase (AST)

In the present study the AST level of unaffected fish was found to be 37.00±2.00 IU/L. But after 6 hrs it increased to 67.00±2.00 IU/L in *Aeromonas hydrophila* inoculated rohu and remained at 37.00±2.00 IU/L when subjected to *Edwardsiella tarda* inoculation at sublethal doses (Table 8; Figure 47). After 7 days it decreased to 34.00±2.00 IU/L in *Aeromonas hydrophila* inoculated fish and 36.00±4.00 IU/L in *Edwardsiella tarda* inoculated ones. There existed significant differences ($P<0.05$) in ALT between control and *Aeromonas hydrophila* inoculated rohu (Table 8; Figure 47).

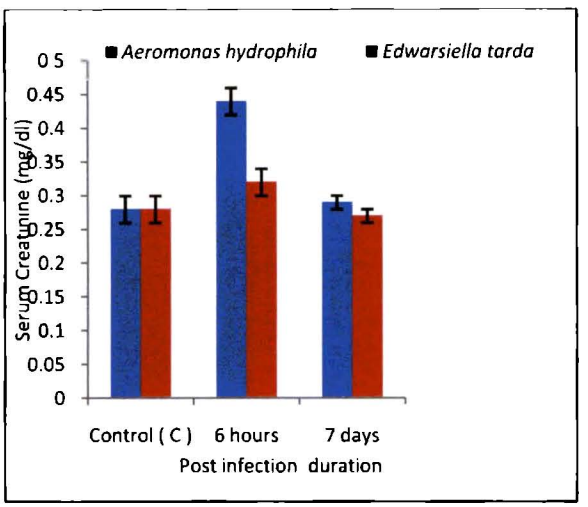

Figure 45

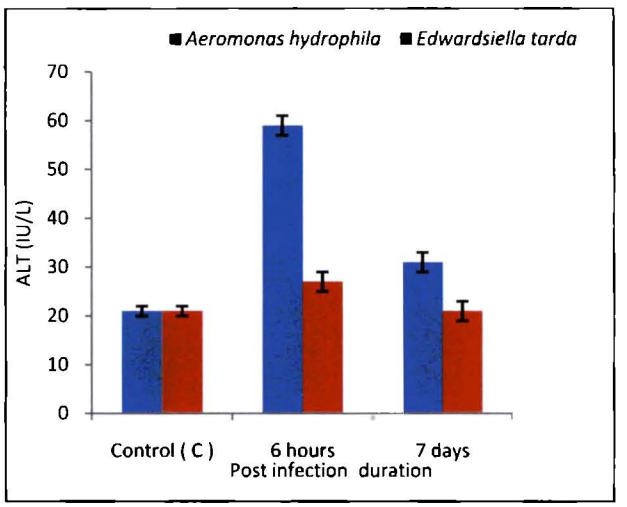

Figure 46

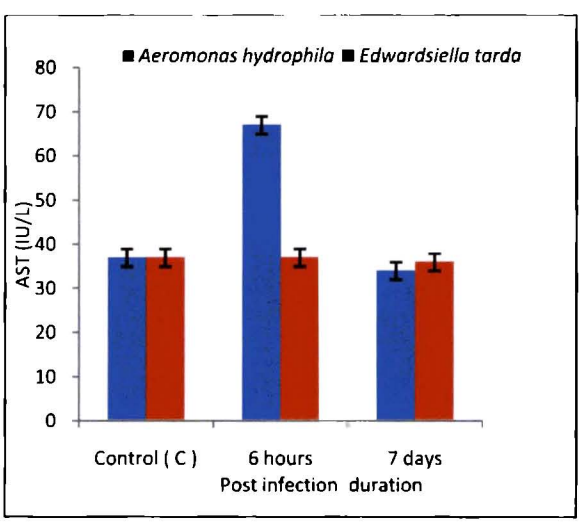

Figure 47

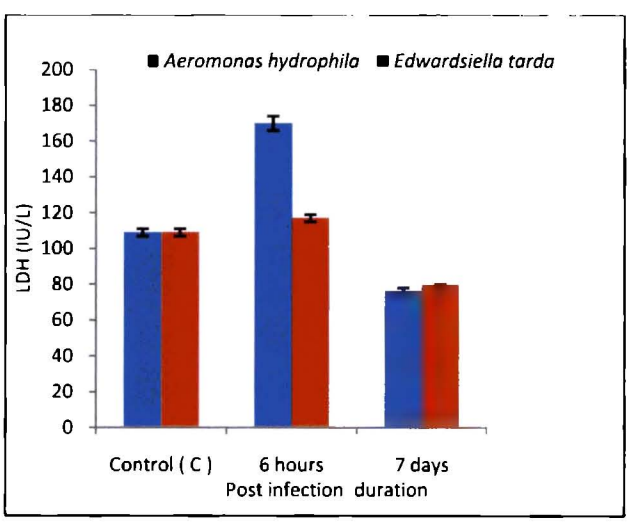

Figure 48

Figure 45. Variation in serum creatinine (mg/dl) of *Labeo rohita* challenged with sublethal doses of *Aeromonas hydrophila* and *Edwardsiella tarda*

Figure 46. Variation in serum alanine aminotransferase (IU/L) of *Labeo rohita* challenged with sublethal doses of *Aeromonas hydrophila* and *Edwardsiella tarda*

Figure 47. Variation in serum aspartate aminotransferase (IU/L) of *Labeo rohita* challenged with sublethal doses of *Aeromonas hydrophila* and *Edwardsiella tarda*

Figure 48. Variation in lactate dehydrogenase (IU/L) of *Labeo rohita* challenged with sublethal doses of *Aeromonas hydrophila* and *Edwardsiella tarda*

4.4.2.8. Lactate dehudrogenase (LDH)

In the present investigation uninfected rohu had LDH level of 109.00 IU/L. But after 6 hrs it increased to 170.00±4.00 IU/L but declined to 76.00±2.00 IU/L when subjected to *Aeromonas hydrophila* inoculation at sublethal dose (Table 8; Figure 48). Likewise, the mean urea level obtained were 6.60±0.60 mg/dl and 5.30±0.60 mg/dl after 6 hours and 7 days of post infection respectively, when rohu was experimentally challenged with *Edwardsiella tarda* at sublethal dose (Table 8; Figure 48).

4.4.2.0. Cortisol

The mean cortisol content of rohu in control (initial) was 19.00±1.00 µg/dl. After 6 hrs it increased to 30.00±2.00 µg/dl, however, after 7 days it decreased to 24.00±1.00 µg/dl when experimentally challenged with *Aeromonas hydrophila* infection at sublethal dose (Table 8; Figure 49). Likewise, the mean cortisol content obtained were 25.00±1.00 µg/dl and 23.00±2.00 µg/dl after 6 hours and 7 days, respectively, when rohu was experimentally challenged with *Edwardsiella tarda* at sublethal dose (Table 8; Figure 49).

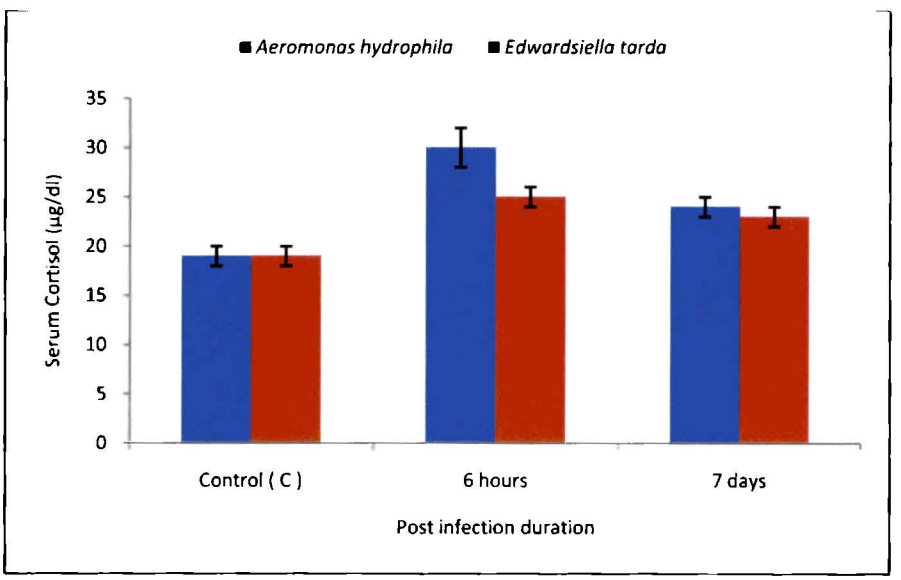

Figure 49. Variation in cortisol (µg/dl) of *Labeo rohita* challenged with sublethal doses of *Aeromonas hydrophila* and *Edwardsiella tarda*

Table 9. Range of water quality parameters recorded in different sets of experiment

Stressor / Parameter	Acute single handling stress (ASHS)	Acute long term handling stress (ALHS)	Transportation procedure				Bacteriological infection	
			Before Transportation (BT)	After transportation (AT)			*Aeromonas hydrophila*	*Edwardsiella tarda*
				AT1 (500 g/bag)	AT2 (1.0 kg/bag)	AT3 (1.5 kg/bag)		
Temperature (°C)	26.50 – 28.00	24.00 – 28.00	28.00	32.00 – 32.50	32.00 – 32.50	32.00 – 33.00	26.00 – 29.00	26.00 – 29.00
Dissolved oxygen (mg/l)	4.50 – 4.80	4.00 – 5.10	3.70	1.30 – 1.50	0.72 – 0.80	0.48 – 0.52	4.20 – 4.80	4.20 – 4.80
pH	7.50 – 7.90	7.50 – 7.90	7.50	7.06 – 7.10	6.85 -7.10	6.80 – 6.90	7.40 – 7.70	7.40 – 7.77

CHAPTER-5

Discussion

5. DISCUSSION

Stress is an unavoidable component in aquaculture practices which is associated with transportation, handling, netting, water and sediment quality, vaccination and disease treatment which causes immunosuppressant and as a result of this fish not only subjected to various physiological changes but also succumb to diseases when exposed to such acute stressors (Mock *et al.*, 1990). In aquaculture practices the understanding of fish stress response is essential to avoid stress-related problems, and to improve fish quality, optimizing productions. Haematological and biochemical parameters have been acknowledged as valuable tools for monitoring fish health, confirming maturation and monitoring any changes in the quality of water and related soil. Information on haematological and biochemical indices of fish, though, very essential for early diagnosis of disease and studying the effect of stress (Wedemeyer and Nelson, 1984), their ranges of normal values of the key biochemical parameters are still undefined for different species in different aquaculture conditions. In the present study haematological and biochemical changes in rohu (*Labeo rohita*) subjected different stressors were analysed and the results obtained are discussed below.

Transportation of fish seeds from hatchery to farms is stressful, yet it is an inevitable procedure in aquaculture practices. Optimizing the packing density is very much imperative to avoid stress and stress-related mortality. Fish transportation at the packing density of 1.5 kg/bag (200 g/l) from Naihati market is the general practice. Though the abrasion in polythene bags during transportation was not quantified in the present study, but was evident in significant differences that were observed in higher packing densities. A cumulative of 30% mortality was recorded during and after transportation in fish transported at the rate of 1.5 kg/bag, however, there was no such mortalities in other two packing densities.

In general all the water quality parameters analysed in different sets of experiment were within the tolerance limits of carps. The water temperature recorded (24.00 – 33.00°C) during the present study was well within the conducive limits for the growth of *Labeo rohita* fingerlings. Carps are reported to thrive well between 18.3 and 37.8°C (Jhingran, 1982). Water pH observed in the present study ranged from a minimum of 6.80 to a maximum of 7.90 which is also well within the acceptable limits for carps, which are known to thrive well in pH between 6.70 – 8.10 (Victor and Connors, 2004; Tuladhar, 2003) found that pH range within 6.7 – 8.1 has given good result regarding the growth studies of *Catla catla*. The dissolved oxygen content varied from 4.20 to 5.10 mg/l in different sets of experiment except during transportation procedure. These

values are well within the prescribed limits of 3.6 to 6.0 mg/l (Victor *et al.*, 2004). Dissolved oxygen content was 3.70 mg/l before transportation which was reduced to 1.30 – 1.50 mg/l, 0.72 – 0.80 mg/l and 0.48 – 0.52 mg/l in AT1 (500 g/bag packing density), AT2 (1 kg/bag packing density) and AT3 (1.5 kg/bag packing density) respectively. Aravindakrishnan *et al.* (2011) have reported decrease in dissolved oxygen from an initial value of 5.6 mg/l to 3.4 mg/l, 5.4 mg/l to 1.56 mg/l, 7.2 mg/l to 0.22 mg/l in waters where rohu (*Labeo rohita*), catla (*Catla, catla*) and mrigal (*Cirrhinus mrigala*) were transported for one hour. From the present study it was clear that the fish in the packing density of 1.5 kg/bag consumed large amount of dissolved oxygen due to greater demand for increased metabolic rate.

5.1. Haematological parameters

5.1.1. Total erythrocyte count (TEC) or Red blood corpuscles (RBC)

Total erythrocytes or Red blood corpuscles (RBC) play an important role in transportation of energy to the cells in terms of oxygen and removing carbon dioxide from the body. The TEC makes up the majority of the blood cells. Fish RBC are nucleated and have life span of about 1.5 yrs (Bowser, 1993).

5.1.1.1. Acute single handling stress

The mean total erythrocyte count in the unstressed *Labeo rohita* (control) was $1.15\pm0.05\times10^6$ no/ml which increased significantly due to handling procedure. The TEC values were found to be $1.30\pm0.00\times10^6$ no/ml, $1.45\pm0.05\times10^6$ no/ml and $2.00\pm0.01\times10^6$ no/ml when fish were subjected to 5 min (ASHS1), 10 min (ASHS2) and 15 min (ASHS3) acute single handling stress, respectively. There existed significant differences ($P<0.05$) in TEC values between control and ASHS3 group, ASHS1 and ASHS3 groups and ASHS2 and ASHS3 groups.

5.1.1.2. Acute long term handling stress

The mean TEC was found to be $1.17\pm0.01\times10^6$ in *Labeo rohita* subjected to acute long term stress for 5 min (ALHS1) daily. It increased to $1.18\pm0.02\times10^6$ no/ml of blood when subjected for 10 min (ALHS2). The highest value of 1.99×10^6 no/ml of blood was recorded when subjected for 15 min (ALHS3). Significant differences ($P<0.05$) were noticed between unstressed (control) group and fish subjected for 10 min and 15 min acute long term stress as well as between ALHS1 and ALHS3, and ALHS2 and ALHS3 groups. The results corroborated

with the findings of Sadler *et al.* (2000) who reported increased RBC count in Atlantic salmon *Salmo salar*. The RBC count of unstressed female remained at 0.94×10^6 no/ml of blood, which was increased to 0.97 $\times10^6$ no/ml of blood, due to handling stress, However, they opined that RBC count does not show any significant impact in case of short term acute stress. Aberu *et al.* (2009) also found that in pacu (*Piaractus mesopotamicus*) RBC count increased from 2.30×10^6 no/ml of blood in control group to 2.40×10^6 no/ml of blood, due to 5 min acute handling. The mean RBC count in jundia (*Rhamdia quelen*) was significantly higher in stressed fish (1.70×10^6 no/ml of blood) than control (1.5×10^6 no/ml of blood) (Leonardo *et al.*, 2003). Contrary to the present study, Gobore *et al.* (2006) found in *Clarias gariepinus* the RBC count decreased from 0.73×10^6 no/ml to 0.70×10^6 no/ml of blood and in *Tilapia zilli* the RBC count decreased from 1.10×10^6 no/ml to 1.05×10^6 no/ml when they are subjected to handling stress.

5.1.1.3. Experimentally challenged bacterial infection stress

In the present investigation the mean TEC value of control *Labeo rohita* (initial) was $1.26\pm0.03\times10^6$ no/ml of blood. After 6h the TEC increased to $1.40\pm0.04\times10^6$ no/ml of blood and after 7 days it further increased to $1.45\pm0.01\times10^6$ no/ml of blood when subjected to *Aeromonas hydrophila* challenge at sublethal dose. Likewise, the TEC values in *Labeo rohita* increased to $1.32\pm0.01\times10^6$ no/ml of blood and $1.42\pm0.00\times10^6$ no/ml of blood when subjected to *Edwardsiella tarda* challenge at sublethal dose for 6h and 7 days, respectively. The TEC values, however, did not differ significantly ($P>0.05$) between control and after 6h in both the cases. There was a significant difference ($P<0.05$) between control and 7 days post-challenge groups in both the cases. The difference between the 6h and 7 days post-*Edwardsiella tarda* challenged groups was also significant. Likewise, in Nile tilapia (*Oreochromis niloticus*) the RBC increased from 1.02 $\times10^6$ no/ml of blood to 1.09×10^6 no/ml of blood when experimentally injected with sublethal dose of *Aeromonas hydrophila* (Bailone *et al.*, 2010). According to Pourgholam *et al.* (2013), when grass carp (*Ctenopharyngodon idella*) was subjected to *Aeromonas hydrophila* challenge at sublethal dose for a long duration, the RBC count decreased from 1.80×10^6 no/ml of blood to 1.70×10^6 no/ml of blood, which is not in accordance to the present finding. Phanikumar *et al.* (2013) observed that when pangus (*Pangasianodon hypophthalmus*) were subjected to *Aeromonas hydrophila* challenge at sublethal dose for a long duration the RBC count decreased from 4.66×10^6 no/ml of blood to 4.21×10^6 no/ml of blood, which they have attributed to hypochromic microcytic anaemia. Decreased RBC values also indicated that such cells are being destroyed by the leukocytosis activity. In rainbow trout (*Oncorhynchus mykiss*) the RBC counts decreased from 1.00×10^6 no/ml of blood to 0.50×10^6

no/ml of blood when subjected to *Aeromonas hydrophila* challenge at lethal dose for long duration (Rehulka *et al.*, 2002).

The elevated red blood cells in the circulation (erythropoesis), is presumably a first mechanism through which fish might compensate for higher metabolic rate which invariably prevails in stressed conditions (Wepener *et al.*, 1992). A second mechanism by which fish might compensate for poor oxygen uptake during stress conditions is via the release of a large number of mature red blood cells in the general circulation. This is thought to be stimulated by b-adrenergic action on the hemopoietic tissues, which contract and release stored mature red cells (Wepener *et al.*, 1992). This mechanism might, however, compensate for short term oxygen concentration variations in blood or water (Nespolo and Rosenmann, 2002). The spleen is a small organ in relation to body mass in teleosts, yet it provides an appreciable reservoir of erythrocytes which, under the control of the autonomic nervous system, may be released rapidly into the circulation (Fange and Nilsson, 1985). Adjustments to blood characteristics are very common when fish are exposed to stress which includes adrenergically mediated red blood cell swelling and release of red blood cells from the spleen (Val, 1993; Randall and Perry, 1994) as reported in tambaquii, *Colossoma macropomum* (Moura, 1994) and also when fish are asphyxiated (Bonnet, 1929; Yamamoto *et al.*, 1985) and hence may acclaim for the increase in RBC as observed in the present investigation. Generally, red blood cells undergo premature destruction by intravascular haemolysis in circulation. This may be caused by trauma to the RBC, either by natural complement activation process to the RBC or by exogenous toxins (Cooper and Bunn, 1988). The gradual shrinkage and ultimate disintegration of RBC might have contributed to the poorer value of RBC count and Ht values (Homechaudhuri *et al.*, 1986). Removal of dysfunctional erythrocytes from the blood circulation due to oxygen shortage might also have also caused the reduction in TEC as observed by some authors (Gbore *et al.*, 2006; Phanikumar *et al.*, 2013).

5.1.2. Total leukocyte count (TLC) or White blood corpuscles (WBC)

The white blood corpuscles (WBC) perform the most vital role by preventing from any external stress as it associated with cellular immunity and with the production of humoral antibody (Wedemeyer *et al.*, 1990). Leukocytes are involved in the regulation of immunological function and their numbers increase as protective response in fish to stress. Their counts in fish blood are considerably higher than that of a man or other vertebrates (Andrews, 1965).

5.1.2.1. Acute single handling stress

In the present study, the mean total leukocyte count in the unstressed fish (control) was $51.50\pm0.05\times10^3$ no/ml, which showed an increasing trend with acute and chronic handling duration. The mean TLC values in *Labeo rohita* subjected to acute single handling stress (ASHS) for 5 min (ASHS1), 10 min (ASHS2) and 15 min (ASHS3) were found to be $85.00\pm02.00\times10^3$ no/ml, $91.50\pm0.05\times10^3$ no/ml and $99.00\pm2.00\times10^3$ no/ml, respectively. There existed significant differences ($P<0.05$) in TLC values between control and all the stressed groups, ASHS1 and ASHS3 groups and ASHS1 and ASHS3 groups.

5.1.2.2. Acute long term handling stress

The mean TLC values in *Labeo rohita* subjected to acute long term stress for 5 min/day (ALHS1), 10 min/day (ALHS2) and 15 min/day (ALHS3) were recorded as $52.00\pm1.00\times10^3$ no/ml, $56.00\pm2.00\times10^3$ no/ml and $59.00\pm3.00\times10^3$ no/ml, respectively. However, there was no significant differences ($P>0.05$) between control and stressed fish as well as among the stressed fish.

On the other hand, the mean WBC count in jundia (*Rhamdia quelen*) was significantly higher in stressed fish (3.20×10^3 no/ml of blood) than control (2.90×10^3 no/ml of blood) (Leonardo *et al.*, 2003). Gbore *et al.* (2006) found in *Clarias gariepinus* the WBC count increased from 1.29×10^3 no/ml of blood to 4.20×10^3 no/ml of blood and in *Tilapia zilli* the WBC count increased from 1.72×10^3 no/ml to 1.80×10^3 no/ml when they were subjected to handling stress and their findings are in concurrence with the present result.

5.1.2.3. Experimentally challenged bacterial infection stress

The mean TLC value in control *Labeo rohita* (initial) was $44.00\pm2.00\times10^3$ no/ml of blood. In 6h post-*Aeromonas hydrophila* challenge group, it increased to $64.00\pm3.00\times10^3$ no/ml of blood. However, in 7 days post-*Aeromonas hydrophila* challenge group, it decreased to $46.50\pm0.50\times10^3$ no/ml of blood. The mean TLC values obtained were $49.50\pm0.50\times10^3$ no/ml of blood and $41.20\pm1.50\times10^3$ no/ml of blood after 6h and 7 days, respectively, in *Edwardsiella tarda* challenged *Labeo rohita*. The TLC values differed significantly ($P>0.05$) between control and in 6h post-challenged *Labeo rohita* as well as 6h and 7 days post-*A. hydrophila* challenged rohu. However, there was no significant difference between ($P>0.05$) between control and 7 days post-challenged *Labeo rohita*. However, the difference in TLC values between 6 hours and 7 days post-*Edwardsiella tarda* challenged rohu was significant ($P<0.05$). According to Pourgholam *et al.* (2013), when grass carp (*Ctenopharyngodon idella*) were subjected to

Aeromonas hydrophila challenge at sublethal dose for a long duration the WBC count increased from 1.90×10^3 no/ml of blood to 2.70×10^3 no/ml of blood, which upholds the present findings. Similarly, Phanikumar *et al.* (2013) also reported that when pangus (*Pangasianodon hypophthalmus*) are subjected to *Aeromonas hydrophila* infestation at sublethal dose for a long duration the WBC count increased from 3.91×10^3 no/ml of blood to 6.05×10^3 no/ml of blood. Likewise, Bailone *et al.* (2010) found in Nile tilapia (*Oreochromis niloticus*) when experimentally challenged with sub lethal dose of *Aeromonas hydrophila,* WBC count increased from 18.19×10^3 no/ml of blood to 37.66×10^3 no/ml of blood, and their report also concurred with present result. Sopinska (1984) observed increase in leukocytes from $40.51\pm 10.65 \times 10^3$ no/mm to $72.16\pm 7.48X\ 10^3$ no/mm in carps subjected to transportation stress.

The TLC may increase with stress as they are involved in the regulation of immunological function and as a protective response stress in fishes (Hymarathi and Rao, 1999; Santhakumar *et al.*, 1999; Nussey *et al.*, 2002). The reports of Wlasow and Dabrowska (1990) and Svobodova *et al.* (1999) showed a decrease in leukocyte count in fish exposed to stress. Stressors induce the secretion of adrenocorticotropic hormone (ACTH) stimulating the adrenal cortex activity (Fryer, 1975). The result is the mobilization of protective mechanisms including the leukocyte system of the body (Sopinska, 1984). Increased leukocytes seem to be the result of an alarm reaction occurring during stress as observed in the present study, which is also confirmed by results obtained by Weinreb (1958).

The stress induced elevation of plasma cortisol has a direct cytolytic effect on lymphocytes (Wiiki *et al.*, 1989; Engelsma *et al.*, 2003). Lymphocytopoenia in stressed fish might occur due to the extravasations of the cells and their penetration into the epithelium of gills, skin or intestine. The movement of immune cells during stressful periods is influenced by stress hormones; therefore, the mobilization of neutrophils and macrophages that form the first line of defense may be important for survival (Ruane *et al.*, 2002).

In experimentally challenged *Labeo rohita*, the TLC was found to be decreased from initial values to 7 days post-challenge, probably due to prolonged stress leading to impairment in leucopoiesis resulting in reduction of TLC. Probably the immune system initially stimulated leukocyte production (Hymarathi and Rao, 1999; Nussey *et al.,* 2002) to compensate the WBC loss, resulting in increased number in later days. Pourgholam *et al.* (2013) attributed increase in leukocytes in *A. hydrophila* inoculated grass carp *Ctenopharyngodon idella* to increase in the levels of respiratory burst.

5.1.3. Haemoglobin

The transport of oxygen from the site at which it is acquired to the remaining parts of the body in which it is needed is the primary function of the blood pigment, haemoglobin (Hb).

5.1.3.1. Acute single handling stress

In the present study there was an irregular trend in haemoglobin content of *Labeo rohita* subjected to acute single handling stress. The mean haemoglobin value in the unstressed (control) fish was 3.80±0.20 g/dl. Maximum haemoglobin content (4.10±0.00 g/dl) was recorded in ASHS3 (fish subjected to 15 min acute single handling stress); whereas minimum value (3.10±0.10 g/dl) was found to be in ASHS1 (fish subjected for 5 min acute single handling stress). An intermediate value of 3.80±0.20 g/dl was noticed in ASHS2 (fish subjected to 10 min acute single handling stress). There existed significant differences ($P<0.05$) in haemoglobin content between control and AHSS3 groups, ASHS1 and ASHS3 groups.

5.1.3.2. Acute long term handling stress

There was an increased trend in haemoglobin content of *Labeo rohita* subjected to acute long term handling stress. Fish subjected to 5 min acute long term handling stress (ALHS1) had mean haemoglobin level of 3.90±0.10 g/dl which increased to 4.20±0.10 g/dl and 4.25±0.00 g/dl in cases of fish subjected to 10 min (ALHS2) and 15 min (ALHS3) acute long term handling stress, respectively. The Hb values, however, did not differ significantly ($P>0.05$) between control and all other stressed group, as well as among the stressed groups.

The present study validated the findings of Sadler *et al.* (2000) who reported a similar trend in the case of Atlantic salmon (*Salmo salar*) where the haemoglobin level of unstressed female was 8.37 g/dl, which then decreased to 7.97 g/dl and further dropped to 6.88 g/dl. However, its level increased to 7.10 g/dl at later stages. This implied that haemoglobin may not show any significant impact in case of short term acute stress. Such an irregular trend was also observed by Aberu *et al.* (2009) in pacu (*Piaractus mesopotamicus*). In their study, they have recorded the Hb level of 12.50 g/dl in unstressed (control) fish, which increased to 12.80 g/dl due to 5 min acute handling and then it reduced to 12.00 g/dl when fish was subjected to 15 min handling stress. Leonardo *et al.* (2003) did not find any significant change in haemoglobin concentration in jundia (*Rhamdia quelen*) due to short term acute stress, which further established the fact that short term acute stress may not have impact on haemoglobin content of blood.

5.1.3.3. Transportation stress

Haemoglobin content showed significant variation with transportation stress as well as density of packing in the present investigation. The mean Hb value in fish of BD group (before disturbance – control) was 3.80±0.02 g/dl and it increased to 4.05±0.15 g/dl before transportation (BT). The mean Hb values further increased to 5.40±0.00 g/dl in 500 g/bag packing density (AT1), 4.90±0.02 g/dl in 1.0 kg/bag packing density (AT2) and 5.20±0.02 g/dl in 1.5 kg/bag packing density (AT3) immediately after transportation. The haemoglobin content differed significantly ($P< 0.05$) between BT and AT1, AT2 and AT3 groups. However, there was no significant differences ($P>0.05$) among the packing densities immediately after transportation.

The result obtained by Dobsikova *et al.* (2006) in case of common carp (*Cyprinus carpio*) showed increase in haemoglobin from 3.20 g/dl to 3.62 g/dl when subjected to 12h transportation stress. Similar findings were also observed by Dobsikova *et al.* (2009) in case of common carp (*Cyprinus carpio*) where Hb value of control fish was 102.00 g/L, which increased to 103.54 g/L due to 12 h transportation. Sopinska (1984) also reported increase in Hb value of carp blood from 7.83 mg/dl to 8.01 mg/dl, when carps are subjected to transportation stress. However, Anthony *et al.* (1961) found a decreased in the Hb level in *Tilapia guinensis* from 5.33 mg/dl to 3.76 mg/dl when subjected to acclimation after capture, which deviates from the other reports and the present findings.

5.1.3.4. Experimentally challenged bacterial infection stress

In the present study the haemoglobin level of control *Labeo rohita* (initial) was 4.00±0.20 g/dl of blood. In 6h post-*Aeromonas hydrophila* challenge group, at sublethal dose, the value decreased to 3.80±0.00 g/dl and in 7 days post-challenge group it increased to 4.10±0.20 g/dl. The haemoglobin concentration values in *Labeo rohita* initially increased to 4.10±0.20 g/dl 6h post-*Edwardsiella tarda* challenge; while it decreased to 4.00±0.00 g/dl in 7 days post-*Edwardsiella tarda* challenge group. However, there was no significant differences ($P>0.05$) in haemoglobin between control and stressed fish as well as among the stressed fish also for both *Aeromonas hydrophila* challenged and *Edwardsiella tarda* challenged *Labeo rohita*. According to Pourgholam *et al.* (2013), when grass carp (*Ctenopharyngodon idella*) was subjected to *Aeromonas hydrophila* challenge at sublethal dose for a long duration, the Hb level decreased from 5.4 g/dl to 4.6 g/dl which corroborate the present findings. Similarly, Phanikumar *et al.* (2013) also observed decrease in haemoglobin content from 14.03 g/dl to 12.11 g/dl in pangus (*Pangasianodon hypophthalmus*) subjected to *Aeromonas hydrophila* challenge at sublethal

dose for a long duration. Correspondingly, Rehulka *et al.* (2002) also recorded a similar trend (reduction in Hb from 6.00 g/dl to 4.20 g/dl) in rainbow trout (*Oncorhynchus mykiss*) when experimentally challenged with *Aeromonas hydrophila* at sub lethal dose. Furthermore, Jin ha Yu (2010) experimentally challenged *Silurus asotus* with *Edwardsiella tarda* and found that haemoglobin reduced from 3.25 g/dl to 1.60 g/dl after 48h injection.

Usually haemoglobin concentration seems to be less influenced by acute stress. Changes in the haemoglobin content of blood in response to the environment might be either due to change in the number of erythrocytes or due to change in the haemoglobin concentration of the individual cells. It has been shown that fishes produce haemoconcentration, as a strategy for increasing oxygen carrying capacity of blood during stress conditions or during high energy demand. Anthony (1961) suggested that the oxygen carrying capacity of cell might increase due to elevation in metabolic rate under stress condition through enhanced haemoglobin content and haemoglobin-oxygen affinity. Increased oxygen carrying capacity, based on direct measurement or inferred from elevated haemoglobin, has also been noted in a number of fish including chronically hypoxic killifish (Greaney and Powers, 1978), trout (Swift and Lloyd, 1974; Soivio *et al.,* 1980) and eels (Wood and Johansen, 1972). The decreased Hb trend may be a result of swelling of RBC as well as poor mobilization of haemoglobin from spleen to other hemopoeitic organs (Scott *et al.,* 2007).

5.1.4. Haematocrit

Haematocrit refers to the ratio of erythrocytes to plasma in the blood and measures the packed cell volume of the erythrocytes contained in the blood or measure of cellular fraction in the blood and expressed as a percentage of plasma volume (Ht %). There exists a positive correlation between the haematocrit value and the total erythrocyte count of the fishes. Any increase in haematocrit may indicate stress induced spleenic release of red blood cells. The blood haematocrit in the present study showed a significant variation with acute handling periods in comparison to control.

5.1.4.1. Acute single handling stress

The mean haematocrit value in the unstressed *Labeo rohita* (control) was comparatively lower (14.20±0.3%) and increased with the duration of acute single handling stress, i.e. it was

22.00±1.00% for 5 min (ASHS1), 24.50±1.10% in case of 10 min (ASHS2) and 26.25±0.75% in case of 15 min (ASHS3). There were significant differences (P <0.05) in haematocrit values between control and each of the stressed group. The differences among the stressed groups was, however, insignificant (P>0.05).

5.1.4.2. Acute long term handling stress

The mean haematocrit values recorded in the present study were found to be 11.09±037%, 13.99±0.33% and 16.95±0.00% in *Labeo rohita* subjected to 5 min (ALHS1), 10 min (ALHS2), and 15 min (ALHS3) acute long term handling stress, respectively. Haematocrit values differed significantly (P<0.05) between control and each of the stressed group, as well as between ALHS1 and ALHS2, ALHS1 and ALHS3 and ALHS2 and ALHS3 groups. The present results are in concurrence with the findings of Sadler *et al.* (2000) who reported increase in Ht level of Atlantic salmon (*Salmo salar*) from 32.50% (unstressed) to 35.70% due to handling stress. Similarly, Aberu *et al.* (2009) found that pacu (*Piaractus mesopotamicus*) had higher Ht level (36.90%) when subjected to 5 min acute handling stress than that of unstressed (34.50%) one. Giulia *et al.* (2008) recorded significantly higher mean Ht level in stressed sea bass *Dicentrarchus labrax L.* (35.00%) than control (25.00%). On the other hand, Leonardo *et al.* (2003) did not find any significant change in haematocrit value in jundia (*Rhamdia quelen*) due to short term acute stress.

5.1.4.3. Transportation stress

The present investigation also implied a significant variation in haematocrit value with transportation procedures. The mean haematocrit value in the undisturbed fish (BD) was 14.20%, which increased significantly as the transportation procedure begun. Before transportation (BT) it was 18.93%, which increased to 27.82%, 34.96%, and 36.93% in the fish packed at the rate of 500 g/bag (AT1), 1 kg /bag (AT2) and 1.5 kg/bag (AT3). The haematocrit values did not differ between AT2 and AT3 groups (P>0.05).

A significant increase in Ht level was also reported in juvenile tambaqui (*Colossoma macropomum*) where Ht level in undisturbed fish was 25.00%, which was increased to 30.00% before transportation and further it increased to 32.00%, 34.00%, 32.00% and 32.00% immediately after transportation and then decreased to 23.00%, 28.00%, 27.00% and 26.00% after 24h of transportation in fishes transported at 78 kg/cubic meter,156 kg/cubic meter, 234 kg/cubic meter and 312 kg/cubic meter packing densities, respectively (Gomes *et al.*, 2003).

Dobsikova *et al.* (2009) reported that in case of common carp (*Cyprinus carpio*) where Ht value of control fish was 42.00% which remained same in case of 7 h and increased to 46.00% after 12 h transportation. The results of the present study also coincided with the findings of Dobsikova *et al.* (2006) who noticed an increment in Ht value from 32.00% to 36.00% in common carp (*Cyprinus carpio*) when subjected to 12 h transportation stress. Sopinska (1984) also documented increased haematocrit value from 35.80% to 49.80%, when carps were subjected to transportation stress. However, Aberu *et al.* (2008) did not observe any variation in Ht in the case of juvenile matrinxa (*Brycon amazonicus*) subjected to 4h transportation stress rather it decreased to 32.00% after 24h of transportation.

5.1.4.4. Experimentally challenged bacterial infection stress

The mean haematocrit value in control *Labeo rohita* (initial) was 15.00%. After 6 h of challenge with *Aeromonas hydrophila* at sublethal dose it increased to 24.80%; however, after 7 days of challenge it decreased to 18.22%. The mean haematocrit values obtained were 24.40% and 23.42% after 6h and 7 days of challenge with *Edwardsiella tarda* at sublethal dose. Bailone *et al.* (2010) observed a decrease in Ht level from 26.33% to 22.33% in Nile tilapia (*Oreochromis niloticus*) when subjected to *Aeromonas hydrophila* challenge at sublethal dose. They argued that this event is occurred due to insufficient inoculation to provoke blood alteration. According to Pourgholam *et al.* (2013), when grass carp (*Ctenopharyngodon idella*) was subjected to *Aeromonas hydrophila* challenge at sublethal dose for a long duration the Ht level decreased from 21.20% to 1.70%. They suggested that alteration in the hematological parameter were brought as an anaemic condition due to decreased synthesis of red blood cells and erythrocytes in the bone marrow when fishes were subjected to long term stress. PhaniKumar *et al.* (2013) observed that when pangus (*Pangasionodon hypopthalamus*) were challenged with *Aeromonas hydrophila* at sublethal dose for a long duration, the Ht level decreased from 49.79% to 35.16%. Similarly, Jin ha Yu (2010) found decreased Ht value in *Silurus asotus* when experimentally injected with subletha lethal dose of *Edwardsiella tarda* from 31.6%- 14.7% after 48 h of injection. These results are in agreement with the present one.

During stress situations, elevated haemoglobin and haematocrit increases the oxygen carrying capacity of blood and, thus, the oxygen supply to major organs in response to higher metabolic demands (Ruane *et al.*, 1999). Most teleosts are oxygen regulators (Hughes, 1973; Rantin and Johansen, 1984), in that they maintain a constant metabolic rate despite a decrease in ambient oxygen saturation that is achieved mainly by increasing the respiratory volume

(Randall, 1982) and adjustments in the oxygen carrying capacity/affinity (Boutilier *et al.*, 1987; Weber and Lykkeboe, 1978) which thereby increases the blood volume.

5.2. Biochemical indices / stress markers

5.2.1. Glucose

Serum glucose level is the most commonly measured indicator of secondary phase stress response in fish (Wedemeyer *et al.*, 1990), which is relatively easy to measure and inexpensive. The increased result of glucose causes as a result of cortisol induced gluconeogenesis, as a result, blood glucose changes are often been used as direct measure of altered cortisol reaction. Blood glucose level may vary according to season and water temperature, and glucose concentration in fish decreases with age and size (Cossinss *et al.* 1982).

5.2.1.1. Acute single handling stress

The mean glucose level in the unstressed *Labeo rohita* (control) was 30.00±1.00 mg/dl which increased significantly due to handling procedure. The glucose levels were found to be 45.00±1.00 mg/dl, 61.00±1.00 mg/dl, 74.50±0.00 mg/dl when fish were subjected to 5 min (ASHS1), 10 min (ASHS2) and 15 min (ASHS3) acute single handling stress, respectively. There were significant differences ($P<0.05$) in glucose levels between control and each of the stressed group as well among the stressed groups.

5.2.1.2. Acute long term handling stress

The mean glucose level was found to be 57.00±2.00 mg/dl, 83.56±0.50 mg/dl and 113.50±1.00 mg/dl in fish subjected to acute long term stress for 5 min (ALHS1), 10 min (ALHS2) and 15 min (ALHS3) daily, respectively. There existed significant differences ($P<0.05$) in glucose levels between control and each of the stressed group as well among the stressed groups. The present results conform with the findings of Svoboda *et al.* (1999) in common carp (*Cyprinus carpio*) where the glucose level of unstressed fish remained at 4.50 mmol/l (81.072 mg/dl) where as it increased to 7.00 mmol/l (126.112 mg/dl) due to short term acute handling (5 min). Abero *et al.* (2009) also found increased glucose level from 58.00 mg/dl to 88.00 mg/dl after 5 min and 90.00 mg/dl after 30 min of acute handling. Serum glucose values were highly variable (25.10-154.00 mg/dl) in juvenile haddock (*Melanogrammus aeglefinus*) exposed to long term handling stress (Hosoya *et al.*, 2006). According to Giulia *et al.* (2008) the glucose level

increased from 60.00 mg/dl to 120.00 mg/dl in *Dicentrachus labrax* due to short term acute stress.

5.2.1.3. Transportation stress

Glucose level varied with transportation stress as well as density of packing in the present research. The mean glucose level in fish of BD group (before disturbance – control) was 30.00±2.00 mg/dl, which increased to 41.00±3.00 mg/dl before transportation (BT). The mean glucose values further increased to 52.50±3.50 mg/dl in 500 g/bag packing density (AT1), 59.00±2.00 mg/dl in 1.0 kg/bag packing density (AT2) and 62.00±1.00 mg/dl in 1.5 kg/bag packing density (AT3) immediately after transportation. Serum glucose showed significant differences ($P<0.05$) between BT and AT2 as well as BT and AT3 groups. Dobsikova *et al.* (2006) noticed increase glucose levels from 8.20 mmol/l (147.73 mg/dl) to 8.70 mmol/l (156.74 mg/dl) and 9.40 mmol/l (169.35 mg/dl) in common carp when transported for the durations of 7h and 12h, respectively. The present results also in agreement with the findings of Abreu *et al.* (2008) recorded on juvenile matrinxa (*Brycon amazonicus*) where glucose level increased from 45.00 mg/dl to 102.00 mg /dl when subjected to 4h of transportation stress. A significant increase in glucose level was also found in juvenile tambaqui (*Colossoma macropomum*) where glucose level in unstreessed fish was 100.00 mg/dl, which increased to 125.00 mg/dl before transportation and further it increased to 140.00 mg/dl, 200.00 mg/dl and 220.00 mg/dl after transportation and then decreased to 80.00 mg/dl, 140.00 mg/dl, 135.00 mg/dl after 24h of transportation in packing densities of 78 kg/cubic meter, 156 kg/cubic meter, 234 kg/cubic meter and 312 kg/cubic meter, respectively (Carlos *et al.*, 2003). Similar results were also obtained by Chatterjee *et al.* (2009) in rohu fry (*Labeo rohita*) where glucose value of control fish was 9.00 mg/dl, which increased to 12 mg/dl and 18 mg/dl after 12h of transportation, and 14 mg/dl and 25 mg/dl after 24h of transportation and 26 mg/dl and 38 mg/dl after 36h of transportation when packed in 40 g/l and 80 g/l packing densities, respectively.

5.2.1.4. Experimentally challenged bacterial infection stress

The present study indicated that glucose level of unstressed *Labeo rohita* (control) which was 68.00±2.00 mg/dl, which increased to 73.00±1.00 mg/dl after 6h of challenge and then decreased to 71.50±1.50 mg/dl after seven days of experimental challenge with sublethal dose of *Aeromonas hydrophila*. Similar trend was observed in *Edwardsiella tarda* infected (sublethal level) rohu in which glucose level rose to 70.00±1.00 mg/dl and remained at the same level even after seven days. However, there was no significant difference ($P>0.05$) in glucose levels

between control and stressed fish as well as among the stressed fish in both the cases of *A. hydrophila* and *E. tarda* challenged *Labeo rohita*. Bailone *et al.* (2010) reported similar findings when Nile tilapia (*Oreochromis niloticus*) was subjected to *Aeromonas hydrophila* infection at sublethal dose with the glucose level increasing from 21.91 mg/dl to 34.17 mg/dl.

In suboptimum or stressful conditions (internal or external) the chromaffin cells release catecholamine hormones, adrenaline and non-adrenaline hormone toward blood circulation (Reid *et al.*, 1998). These stress hormones in conjunction with cortisol mobilize and elevate glucose production in fish through glucogenesis and glycogenolysis pathways (Iwama *et al.*, 1999) to cope with the energy demand produced by the stressor for the "fight of fright" reaction. This might be the cause to raise glucose levels in the present study under stressed conditions. The glucose production is mostly mediated by the action of cortisol which stimulates liver gluconeogenesis and also halts peripheral sugar uptake (Wedemeyer *et al.*, 1990). Glucose is then released (from liver and muscle) toward blood circulation and enters into cells through the insulin action (Nelson and Cox, 2005). Umingel (1977) reported that blood sugar has a direct correlation to metabolism. The increase in blood sugar noticed in the present study could be attributed to differences in respiration and activity as pointed out by Ghosh (1987) as the metabolic rate is known to increase at altered normal conditions. Omoregie *et al.* (1990) reported that tilapia showed marked hyperglycemia response to stressed environmental conditions as a result of incomplete metabolism of the blood sugar due to impaired osmoregulation. When fish absorbs little oxygen from the environment, the respiratory metabolism is depressed and, therefore, stored intracellular glycogen is utilized. Under such conditions, the hyperglycemic hormone is released for the degradation of glucose. This glucose leaks into the blood causing hyperglycemia (Bhattacharya *et al.*, 1987). The general opinion is that the 'stress' initiates glycogenolysis in the liver and this results in hyperglycemia (Menten, 1927). Tondon and Joshi (1973) showed that the blood glucose increased rapidly for the first 2h after stress and rose to 190% of the pre-stress value. Hattingh, (1976) found increase of glucose levels up to 170% with concomitant decrease in haemoglobin concentration and haematocrit.

5.2.2. Total protein

Proteins are important structural and functional molecules in the body. The amino acids from proteins may be used for synthesis of new molecules, or may be burned for energy. Healthy rates of protein synthesis require a homeostatically regulated nitrogen balance, which

compares the rate of incorporation of new proteins into tissue to the rate of protein breakdown to supply energy demands.

5.2.2.1. Acute single handling stress

The mean total protein level in the unstressed fish was significantly higher (2.55±0.040 g/dl), which, however, decreased with the duration of handling stress. Mean protein contents were 2.39±0.00 g/dl in fish subjected to 5 min acute single handling stress (ASHS1), 2.44±0.05 g/dl in case of 10 min (ASHS2) and 2.23±0.11 mg /dl in the case of 15 min (ASHS3). There was no significant difference ($P>0.05$) in protein content between control and stressed groups.

5.2.2.2. Acute long term handling stress

Minimum protein content (1.82±0.07 g/dl) was observed in fish subjected to 15 min acute long term handling stress (ALHS3). Whereas the values recorded in 5 min handling stress (ALHS1) and 10 min handling stress (ALHS2) were found to be 2.10±0.10 g/dl and 1.91±0.26 g/dl, respectively. Significant difference ($P<0.05$) in protein content was noticed between control and ALHS3 group. The present result is analogous to Sadler *et al.* (2000), who found a decrease in protein level from 2.37 g/dl to 1.83 g/dl and 1.93 g/dl to 1.40 g/dl due to acute handling in Atlantic salmon (*Salmo salar*). Gbore *et al.* (2006) found decrease in protein content from 2.37 g/dl to 2.21 g/dl in *Tilapia zilli* and from 5.29 g/dl to 5.06 g/dl in *Clarias gariepinus* when subjected to handling stress and their findings also corroborated with the present study. The mean total protein level in jundia (*Rhamdia quelen*) was significantly higher in stressed fish (9 g/dl) than control (5 g/dl), (Leonardo *et al.*, 2003) and this result contradicted our findings and earlier reports. They attributed this fact to mobilization of protein as a substrate for hepatic glucogenesis.

5.2.2.3. Transportation stress

The serum protein altered with transportation procedures as well as density of packing in comparison to undisturbed fishes. The mean total protein level in the unstressed fish (BD) was relatively higher (8.28±0.28 g/dl), which decreased with the stress. It decreased as the transportation procedure proceeded. The serum protein value was 7.53±0.27 g/dl before transportation (BT). Immediately after transportation it further declined to 4.42±0.21 g/dl, 2.37±0.37 g/dl, and 3.81±0.10 g/dl in fish stocked at 500 g/bag (AT1), 1 kg /bag (AT2) and 1.5 kg/bag (AT3), respectively. Serum total protein showed significant differences between BT and each of the groups of AT ($P<0.05$). Similar findings were also observed by Dobsikova *et al.*

(2009) in common carp (*Cyprinus carpio*) where total protein level decreased from 33 g/l to 31 g/l when subjected to 7 h and 12 h transportation stress, respectively. Whereas the present result did not corroborate the finding of Dobsikova *et al.* (2006) recorded in common carp (*Cyprinus carpio*) where total protein level increased from 29.8 g/l to 31.1 g/l when subjected to 7h transportation stress.

5.2.2.4. Experimentally challenged bacterial infection stress

The mean protein content of *Labeo rohita* control (initial) was 3.18±0.18 g/dl. After 6h of challenge with *Aeromonas hydrophila* infection at sublethal dose it decreased to 2.49±0.49 mg/dl, however, after 7 days of challenge it increased to 2.89±0.07 mg/dl. Likewise, the mean protein contents of rohu when infected with *Edwardsiella tarda* at sublethal dose were 3.15±0.15 mg/dl and 3.31±0.05 mg/dl after 6h and 7 days of challenge, respectively. Rehulka et al. (2002) noticed a decline in protein level from 35 g/dl to 12 g/dl in rainbow trout (*Oncorhynchus mykiss*) subjected to *Aeromonas hydrophila* infection at sublethal dose for long duration. In the same way, Jin Ha Yu (2010) found in *Silurus asotus* experimentally injected with sublethal dose of *Edwardsiella tarda* the total protein value decreased from 3.01 mg/dl to 2.69 mg/dl after 48 hof injection.

Amino acids are the most important anabolic nutrient, and can be used to synthesize structural and functional proteins of the body. Before amino acids can be oxidized for energy, they must have the amine group removed, a process called deamination. The deaminated amino acid molecule is converted to pyruvic acid, or a Krebs cycle ketoacid intermediate. Deaminated amino acids may also be reconverted to cortisol and contribute to gluconeogenesis. An increase in serum protein might be due to caused by a shift of fluid from the serum to the intracellular compartment and a decrease can be caused by hydration in serum due to osmotic imbalance between extracellular and intracellular compartments (Milligen and Wood, 1982). The reduction in total protein can also be ascribed to its increased catabolism caused by inflammation by infectious diseases (Oehulka and Minaoik, 2007). Protein loss in rohu might also be affected by kidney damage, external lesions and reduction inprotein synthesis in liver (Jin ha Yu, 2010).

5.2.3. Triglycerides

Triglycerides are the storage and transportation system for lipids in the blood.

5.2.3.1. Acute single handling stress

The mean triglyceride content in the unstressed *Labeo rohita* (control) was 24.00±2.00 mg/dl. It increased with the duration of handling stress. Maximum triglyceride (46.50±1.00 mg/dl) was recorded in ASHS3 (fish subjected to 15 min acute single handling stress) where as ASHS1 (fish subjected for 5 min acute single handling stress) had 41.00±0.00 mg/dl and an intermediate value of 43.00±1.00 mg/dl was noticed in ASHS2 (fish subjected to 10 min acute single handling stress). There were significant differences ($P<0.05$) in triglycerides between control and each of the stressed groups as well as ASHS1 and ASHS3 groups.

5.2.3.2. Acute long term handling stress

Mean triglyceride values recorded in 5 min acute long term handling stress (ALHS1), 10 min acute long term handling stress (ALHS2) and 15 min acute long term stress (ALHS3) were found to be 54.00±2.00 mg/dl, 57.00±4.00 mg/dl and 74.50±1.50 mg/dl, respectively. There were significant differences ($P<0.05$) in triglycerides between control and each of stressed group as well as AHLS1 and AHLS3 groups.

5.2.3.3. Transportation stress

The serum glycerides showed variation with transportation procedures as well as density of packing in comparison to undisturbed fishes. The mean triglcerides in the unstressed fish (BD) was relatively lower (24.00±2.00 mg/dl), which increased with the stress. The serum glycerides value increased to 39.00±3.00 mg/dl before transportation (BT). Immediately after transportation it further increased to 54.00±2.00 mg/dl, 58.50±1.50 mg/dl, and 81.50±1.50 mg/dl in fish stocked at 500 g/bag (AT1), 1 kg /bag (AT2) and 1.5 kg/bag (AT3), respectively. Serum triglycerides showed significant difference between BT and AT2 as well as BT and AT3 groups ($P<0.05$).

5.2.3.4. Experimentally challenged bacterial infection stress

In present study the triglyceride level of uninfected *Labeo rohita* was 54.00±2.00 mg/dl. But after 6h of challenge with *Aeromonas hydrophila* and *Edwardsiella tarda* it increased to 110.00±1.50 mg/dl and 79.00±1.50 mg/dl, respectively. After 7 days of challenge the corresponding values declined to 81.50±1.50 mg/dl and 58.5±1 mg/dl in *Aeromonas hydrophila*

and *Edwardsiella tarda* infected fish. There existed significant differences (P<0.05) between control and stressed fish as well as among the stressed fish also between *Aeromonas hydrophila* and *Edwardsiella tarda* infected *Labeo rohita*. Such trend is comparable with studies of Rehulka *et al.* (2002) who demonstrated initial increase and then decrease in triglycerides in rainbow trout (*Oncorhynchus mykiss*) subjected to *Aeromonas hydrophila* infection at sublethal dose for long duration. They reported a decrease in triglyceride from 0.78 mmol/l (1.408 mg/dl) to 0.47mmol/l (0.846 mg/dl). A disorder of elevated serum triglycerides was considered as secondary response to stress. It may be associated with a predisposition to premature coronary artery disease (www.nutrition.gov; www.nhlbi.hih.gov).

5.2.4. Urea

Urea is a major metabolic product containing ammonia, which is toxic to the body. It must be quickly filtered from the blood by the kidneys and its transport plays a vital role in nitrogen elimination and osmotic homeostasis (Saston, 1984).

5.2.4.1. Acute single handling stress

The mean urea concentration in the unstressed *Labeo rohita* (control) was 3.95±0.50 mg/dl, which, however, increased with the duration of handling stress. The mean urea contents were 5.60±0.60 mg /dl in fish subjected to 5 min acute single handling stress (ASHS1), 6.60±0.60 mg/dl in case of 10 min (ASHS2) and 7.00±1.00 mg /dl in the case of 15 min (ASHS3). Significant differences (P<0.05) in urea were observed between unstressed group and ASHS3 as well as between AHSS1 and AHLSS2, and AHSS1 and AHSS3 groups.

5.2.4.2. Acute long term handling stress

Maximum urea content (6.45±2.00 mg/dl) was observed in fish subjected to 15 min acute long term handling stress (ALHS3). Whereas the values recorded in 5 min handling stress (ALHS1) and 10 min handling stress (ALHS2) were found to be 4.75±0.00 mg/dl and 5.95±0.05 mg/dl, respectively. Significant differences (P<0.05) in urea were observed between unstressed group and fish subjected for 5 min long term acute stress as well as between AHLS1 and AHLSC2, and AHLS1 and AHLS3 groups. The present results are comparable with the findings of Leonardo *et al.* (2003) who recorded significantly higher mean urea level (6 mg/dl) in jundia (*Rhamdia quelen*) than unstressed one (3 mg/dl).

5.2.4.3. Experimentally challenged bacterial infection stress

The mean urea content of *Labeo rohita* control (initial) was 3.95±0.50 mg/dl. After 6h of challenge with *Aeromonas hydrophila* at sublethal dose it increased to 7.00±1.00 mg/dl, however, after 7 days it decreased to 5.60±0.60 mg/dl. Likewise, the mean urea levels obtained were 6.60±0.60 mg/dl and 5.30±0.60 mg/dl 6h and 7 days of post-*Edwardsiella tarda* infection respectively, at sublethal dose. However, no significant difference ($P>0.05$) in urea between control and infected (either with *Aeromonas hydrophila* or *Edwardsiella tarda*) rohu were noticed. Rehulka *et al.* (2002) have shown in rainbow trout (*Oncorhynchus mykiss*) subjected to *Aeromonas hydrophila* infection at sublethal dose for long duration the urea level increased from 0.50 mmol/l (1.40 mg/dl) to 1.80 mmol/l (5.04 mg/dl) and their findings are in accordance with the present one. Urea is transported via the blood to the kidneys and it is integrated to the urinary concentration mechanism in the kidney. It was generally considered that urea was passively transported across biological membranes by diffusion. Lately, specific transporters for urea have been identified in the renal medulla (Kata, 1996;) where urea was transported actively (Kawakami, 1998; Borgnia, 1999). Leung *et al.* (1996) suggested that co-transporters, like the Na+-cortisol co-transporters, behave as urea channels. Urea co-transporters may account for urea re-absorption in the proximal tubule and can have a role in the movement of urea across the serum membranes in the intestine, thyroid gland and brain. This finding suggested that urea transport was coupled with substrate transport provides strong evidence against an osmotic mechanism for substrate-coupled water flow. Thus, it appears that a high blood urea concentration recorded in the present study is likely to be a sign of stress associated with the increase in the cortisol level (Borges *et al.*, 1986).

5.2.5. Creatinine

Creatinine is a protein produced by muscle and released into the blood. The amount produced is relatively stable in a given animal. The creatinine level in the serum is, therefore, determined by the rate at which it is being removed, which is roughly a measure of kidney function. If kidney function fails, the creatinine level will rise.

5.2.5.1. Acute single handling stress

The mean creatinine in the unstressed *Labeo rohita* (control) was 0.330±0.030 mg/dl. Maximum creatinine (0.400±0.020 mg/dl) was recorded in ASHS3 (fish subjected to 15 min acute single handling stress); whereas ASHS1 (fish subjected for 5 min acute single handling stress) had 0.350±0.010 mg /dl and an intermediate value of 0.390±0.000 mg/dl was noticed in ASHS2 (fish subjected to 10 min acute single handling stress). However, there was no significant difference ($P>0.05$) in creatinine between control and stressed groups as well as among the stressed groups.

5.2.5.2. Acute long term handling stress

There was an increased trend in creatinine of rohu subjected to acute long term handling stress too. Fish subjected to 5 min acute long term handling stress (ALHS1) had mean creatinine level of 0.535±0.025 mg/dl mg/dl, which increased to 0.645±0.025 mg/dl and 0.745±0.03 mg/dl in fish subjected to 10 min (ALHS2) and 15 min (ALHS3) acute long term handling stress, respectively. Significant differences ($P<0.05$) in creatinine were noticed between unstressed and fish subjected each of the stressed group as well as between ALHS1 and ALHSC3 groups.

5.2.5.3. Transportation stress

The mean creatinine level in fish of BD group (before disturbance – control) was 0.350±0.030 mg/dl and it increased to 0.390±0.000 mg/dl before transportation (BT). The mean creatinine values further increased to 0.445±0.035 mg/dl in 500 g/bag packing density (AT1), 0.560±0.030 mg/dl in 1.0 kg/bag packing density (AT2) and 0.520±0.030 mg/dl in 1.5 kg/bag packing density (AT3) immediately after transportation. There was no significant difference ($P>0.05$) in creatinine level between BT and AT1. However, significant differences existed ($P<0.05$) between BT and AT2 as well as BT and AT3 groups.

5.2.5.4. Experimentally challenged bacterial infection stress

In the present analysis uninfected *Labeo rohita* had creatinine level of 0.28±0.02 mg/dl. But after 6h of challenge with *Aeromonas hydrophila* at sublethal dose, it increased to 0.32±0.02 mg/dl and then declined to 0.29±0.01 mg/dl after 7 days of challenge. Similar trend was observed in fish experimentally challenged with *Edwardsiella tarda*, wherein corresponding values of 6h and 7 days post-challenge were 0.44±0.02 mg/dl and 0.27±0.01 mg/dl,

respectively. There existed significant difference ($P<0.05$) in creatinine between control and 6h of post-*Aeromonas hydrophila* infection. However, difference was insignificant ($P>0.05$) between and control and 7 days post-infection group. There was no significant difference ($P>0.05$) in creatinine between control and *Edwardsiella tarda* infected rohu. Normally creatinine is generated by skeletal muscle through the breakdown of creatinine phosphate for energy. A specific, saturable, sodium and chloride dependent creatinine transporter responsible for creatinine uptake across the serum membrane has been described for skeletal muscle, heart, smooth muscle, fibroblasts, neurolastoma and astroglia cells, as well as for red blood cells and macrophages (Fitch, 1977; Daly, 1980, 1985).

5.2.6. Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST)

Aminotransferases (alanine aminotransferase, ALT and aspartate aminotransferase, AST) catalyze the interconversion of amino acids and α-ketoacids by transfer of amino groups. They play a role of a link between carbohydrate and protein metabolism. Stress may damage enzyme systems by blocking active sites, immobilization of essential metabolites, modification of membrane structure and its permeability. Changes of aminotransferase activities may also cause the disturbances of Kreb's cycle and decrease the level of cycle intermediates. They are detected in blood serum, in the cytoplasm, mitochondria and in various tissues, the activities of which reflect the cell physiological status (van der Oost, 2003; Martinez-Porchas *et al.*, 2011).

5.2.6.1. Acute single handling stress

In the present study, the minimum ALT level (10.00±1.00 IU/L) was found in the unstressed *Labeo rohita* (control), and it showed an increased trend with acute and chronic handling duration. The mean ALT values in rohu subjected to 5 min (ASHS1), 10 min (ASHS2) and 15 min (ASHS3) acute single handling stress were found to be 28.00±2.00 IU/L, 28.00±2.00 IU/L and 36.00±3.00 IU/L, respectively. There existed significant differences ($P<0.05$) in ALT level between control and each of the stressed groups as well as between ASS2 and ASHS3 groups. In the present study, the lowest level of AST (41.00±2.00 IU/L) was found in the unstressed rohu (control), and it showed an increased trend with acute and chronic handling duration. The mean AST values in rohu subjected to 5 min (ASHS1), 10 min (ASHS2) and 15 min (ASHS3) acute single handling stress were found to be 56.00±3.50 IU/L, 58.00±2.00 IU/L and 60.00±3.00 IU/L, respectively. Significant differences ($P<0.05$) in AST levels between control and each of the stressed groups as well as between ASHS1 and ASHS3, and AHSS2 and AHSS3 groups were noticed.

5.2.6.2. Acute long term handling stress

The mean ALT values in rohu subjected to acute long term stress for 5 min/day (ALHS1), 10 min/day (ALHS2) and 15 min/day (ALHS3) were recorded as 14.00±1.00 IU/L, 33.00±0.00 IU/L and 23.00±0.00 IU/L, respectively. There existed significant differences ($P<0.05$) in ALT level between control and ALHS2, and control and ALHS3. Other differences were insignificant. The mean AST values in rohu subjected to acute long term stress for 5 min/day (ALHS1), 10 min/day (ALHS2) and 15 min/day (ALHS3) were recorded as 44.00±1.00 IU/L, 52.50±0.50 IU/L and 53.00±0.00 IU/L, respectively. There were significant differences ($P<0.05$) in AST levels between control and ALHS2, and control and ALHS3 groups. Other differences were insignificant ($P>0.05$). The results of the present study are comparable with the observations of Leonardo *et al.* (2003), where the mean ALT (30.00 IU/L) and AST (75 IU/L) levels were significantly higher in stressed fish than control jundia, *Rhamdia quelen* (ALT: 20.00 IU/L and AST: 400 IU/L, respectively).

5.2.6.3. Transportation stress

The serum ALT level varied with transportation stress as well as density of packing in the present research. The mean ALT level in fish of BD group (before disturbance – control) was 10.00±1.00 IU/L and it increased to 16.00±0.00 IU/L before transportation (BT). The mean ALT values further increased to 18.00±2.00 IU/L in 500 g/bag packing density (AT1), 28.00±4.00 IU/L in 1.0 kg/bag packing density (AT2) and 32.00±2.00 IU/L in 1.5 kg/bag packing density (AT3) immediately after transportation. The serum ALT showed significant differences between ($P<0.05$) BD and BT2 groups as well as BD and BT3 groups. Similar results were obtained by Dobsikova *et al.* (2009) in common carp (*Cyprinus carpio*) where ALT level increased from 1.17 µkat/l (70.059 IU/L) to 1.60 µkat/l (95.80 IU/L) and 2.12 µkat/l (126.945 IU/L) when subjected to 7 h and 12 h transportation stress, respectively. The present results also corroborated with the findings of Chaterjee *et al.* (2009) in *Labeo rohita* juveniles where ALT level increased from 0.44 µkat/l (26.347 IU/L) to 1.45 µkat/l, (86.826 IU/L) 4.62 µkat/l (276.64 IU/L) and 7.36 µkat/l (440.716 IU/L) when subjected to 12h, 24h and 36h transportation stress, respectively.

The present investigation also implied a significant variation in AST levels with transportation procedures. Mean AST in the undisturbed fish (BD) was low (24.00±2.00 IU/L) and increased as the transportation procedure begun. Before transportation (BT) it was 25.00±1.00 IU/L, and increased to 28.00±0.00 IU/L, 31.00±2.00 IU/L and 35.00±1.00 IU/L in the

fish packed at the rate of 500 g/bag (AT1), 1 kg /bag (AT2) and 1.5 kg/bag (AT3), respectively. Similar findings were reported by Dobsikova *et al.* (2009) in common carp (*Cyprinus carpio*) where AST level increased from 1.5 µkat/l (89.82 IU/L) to 2 µkat/l (119.76 IU/L) and 3.5 µkat/l (209.58 IU/L) when subjected to 7h and 12h transportation stress, respectively. Present results corroborate the finding of Chaterjee *et al.* (2009) in *Labeo rohita* juveniles where AST level increased from 23.05 IU/L to 32.36 IU/L, 46.00 IU/L and 61.01 IU/L when they were subjected to 12h, 24h and 36h transportation stress, respectively. The trend was alike in the experiment conducted by Dobsikova *et al.* (2006) in common carp (*Cyprinus carpio*) where AST level increased from 2.2 µkat/l (131.736 IU/L) to 3.4 µkat/l (203.592 IU/L) when subjected to 7h transportation stress.

5.2.6.4. Experimentally challenged bacterial infection stress

In the present study the mean ALT level of uninfected *Labeo rohita* (control) was 21.00±1.00 IU/L. But after 6 h of experimental challenge with *Aeromonas hydrophila* at sublethal dose it increased to 59.00±2.00 IU/L. However, after 7 days of challenge it decreased to 31.00±2.00 IU/L. Similarly, the mean ALT level increased to 27.00±2.00 IU/L after 6h experimental challenge with *Edwardsiella tarda* at sublethal dose, which then decreased to 23.00±2.00 IU/L after 7 days of challenge. There existed significant difference ($P<0.05$) in ALT levels between control and *Aeromonas hydrophila* infected rohu. The difference in ALT levels between control and *Edwardsiella tarda* infected rohu was insignificant ($P>0.05$). Rehulka *et al.* (2002) have shown in rainbow trout (*Oncorhynchus mykiss*) when subjected to *Aeromonas hydrophila* infestation at sublethal dose for long duration the ALT level increased from 1.83 µkat/l (109.58 IU/L) to 4.90 µkat/l (293.41 IU/L). According to Pourgholam *et al.* (2013), when grass carp (*Ctenopharyngodon idella*) was subjected to *Aeromonas hydrophila* infection at sublethal dose for a long duration the ALT level increased from 6.2 IU/L to 13.2 IU/L which was analogous to the present findings.

In the present study the AST level of unaffected fish was found to be 37.00±2.00 IU/L. But after 6h of experimental challenge with *Aeromonas hydrophila* at sublethal dose it increased to 67.00±2.00 IU/L. The value remained at 37.00±2.00 IU/L when subjected to *Edwardsiella tarda* challenge at sublethal dose. After 7 days of challenge, it decreased to 34.00±2.00 IU/L in *Aeromonas hydrophila* infected fish and 36.00±4.00 IU/L in *Edwardsiella tarda* infected ones. There existed significant differences ($P<0.05$) in AST levels between control and *Aeromonas hydrophila* infectedd rohu. The difference in AST levels between control and *Edwardsiella tarda*

infected rohu was insignificant (P>0.05). Rehulka *et al.* (2002) recorded increased level of AST from 12.11 µkat/l (726.35 IU/L) to 13.74 µkat/l (824.125 IU/L) in rainbow trout (*Oncorhynchus mykiss*) when subjected to *Aeromonas hydrophila* infection at sublethal dose for long duration. According to Pourgholam *et al.* (2013), when grass carp (*Ctenopharyngodon idella*) was subjected to *Aeromonas hydrophila* infestation at sublethal dose, the AST level increased from 50.8 IU/L to 53.2 IU/L which was comparable with the present findings. Similarly, Jin HaYu 2010 recorded increase in AST from 100 IU/L to 400 IU/L and 600 IU/L in 24h and 48h post-*Edwardsiella tarda* challenged *Silurus asotus,* respectively.

The catalytic activity of these serum enzymes when they are subjected stressors are known to increase due to leakage of these enzymes from hepatic cells (Hilmy *et al.*, 1987 and Li *et al.*, 2004) and these enzymes further accelerate synthesis and induction of more enzymes, when fish remains in stress for more duration (Campbell *et al.*, 1984). In the present study activities of two transaminases increased significantly generating higher free aminoacid mobilization, which in turn might have produced glucose to cope up with the stress. It is noteworthy to mention here that glucose level also increased in stressed fish in the present investigation. Higher packing density can also be stressful as observed in the present study. Because of liberation of these enzymes in the blood stream, when the hepatic parenchyma was damaged (Tietz , 1987) the changes in biochemical level under the effect of heavy metal toxicity might result in impairment of energy requiring vital processes and, thus, gives an idea about health of the fish population (Martinez-Porchas *et al.*, 2011). The AST was used as clinical diagnostic tool and it was associated with cell necrosis of the liver and skeletal or cardiac muscle, starvation and lack of vitamin E. The serum ALT was an acute hepatic damage good marker (Coppo *et al.*, 2002). At the other hand, aminotransferase are the organ-specific indicators for stress effects and determination of transaminases in serum has proved in diagnosis of liver damage (Van der Oost *et al.,* 2003).

5.2.7. Lactate dehydrogenase (LDH)

Lactate dehydrogenase also called lactic dehydrogenase (LDH) is an enzyme found in the cells of many body tissues, including the heart, liver, kidneys, skeletal muscle, brain, red blood cells, and lungs. It is responsible for converting muscle lactic acid into pyruvic acid, an essential step in producing cellular energy. Lactic dehydrogenase test is used to detect tissue alterations and as an aid in the diagnosis of anemia, and liver disease (Merrit, 2011).

5.2.8.1. Transportation stress

The present study revealed a variation in mean LDH level with transportation stress as well as density of packing. The mean LDH level in fish of BD group (before disturbance – control) was 71.00±6.00 IU/L and it increased to 133.00±3.00 IU/L before transportation (BT). The mean LDH recorded were 133.00±3.00 IU/L in 500 g/bag packing density (AT1), 134.00±2.00 IU/L in 1.0 kg/bag packing density (AT2) and the highest value of 192.00±2.00 IU/L in 1.5 kg/bag packing density (AT3) immediately after transportation. The serum LDH showed significant difference ($P<0.05$) between BD and each of the BT groups. The trend observed by Dobsikova *et al.* (2009) in common carp (*Cyprinus carpio*) where LDH level increased from 9.5 µkat/l to 12 µkat/l and 13.5 µkat/l when subjected to 7h and 12h transportation stress is analogous with the present one. The present results also coincided with the findings of Chaterjee *et al.* (2009) recorded in *Labeo rohita* juveniles where LDH level increased from 109 IU/L to 234.74 IU/L, 253.88 IU/L and 307 IU/L when they were subjected to 12h, 24h and 36h transportation stress, respectively. Similarly, common carp (*Cyprinus carpio*) showed increased LDH values from 10.2 µkat/l to 14.7 µkat/l when transported for 7h (Dobsikova *et al.*, 2006).

5.2.8.2. Experimentally challenged bacterial infection stress

In the present investigation uninfected rohu had LDH level of 109.00 IU/L. But in 6 h post-*Aeromonas hydrophila* challenged fish, it increased to 170.00±4.00 IU/L and then declined to 76.00±2.00 IU/L in 7 days post-challenge. Significant differences ($P<0.05$) between control and *Aeromonas hydrophila* infected rohu (both after 6h and 24 hours) were noticed. Similar trend was observed in fish experimentally challenged with *Edwardsiella tarda*, wherein corresponding values after 6h and 7 days were found to be 117.00±2.00 IU/L and 79.50±0.50 IU/L, respectively. There was no significant difference ($P>0.05$) between control and *Edwardsiella tarda* infected rohu. Rehulka *et al.* (2002) have shown in rainbow trout (*Oncorhynchus mykiss*) subjected to *Aeromonas hydrophila* infection sublethal dose for long duration that the LDH level decreased from 25.97 µkat/l to 24.9 µkat/l. According to Pourgholam *et al.* (2013), when grass carp (*Ctenopharyngodon idella*) was subjected to *Aeromonas hydrophila* infestation at sublethal dose for a long duration the LDH level increased from 110.8 IU/L to 145.6 IU/L, which was comparable with the present study. The increase of the LDH activity could be explained by the elevation in the anaerobic catabolism of blood cortisol and due to the damage of the liver and muscle tissues (Rui and Zuzuki, 1997; Van-Raaij *et al.*, 1996; Oruc and Uner, 1998 and 1999). When disease or injury affects tissues containing LDH, the

cells release LDH into the bloodstream, where it is identified in higher than normal levels (Schrager, 2001).

5.2.9. Cortisol

Fishes display a wide variation in their physiological responses to stress, which is clearly evident in the serum corticosteroid changes, chiefly cortisol. The characteristic elevation in circulating cortisol during the first hour after an acute disturbance can vary by more than two orders of magnitude among species and genetic history appears to account for much of this interspecific variation.

5.2.9.1. Acute single handling stress

The mean cortisol level in the unstressed *Labeo rohita* (control) was 20.00±2.00 µg/dl which, however, increased with the duration of handling stress. Mean cortisol levels were 30.00±1.00 µg/dl in fish subjected to 5 min acute single handling stress (ASHS1), 53.00±2.00 µg/dl in case of 10 min (ASHS2) and 56.00±0.00 µg /dl in the case of 15 min (ASHS3). There were significant differences ($P<0.05$) in cortisol levels between control and each of the stressed fish as well as then ASHS2 and ASHS3 groups.

5.2.9.2. Acute long term handling stress

Maximum cortisol content (60.00±2.00 µg/dl) was observed in fish subjected to 15 min acute long term handling stress (ALHS3). Whereas the values recorded in 5 min handling stress (ALHS1) and 10 min handling stress (ALHS2) were found to be 26.00±1.00 µg/dl and 29.00±1.00 µg/dl, respectively. There existed significant differences ($P<0.05$) in cortisol levels between control and each of the stressed groups. The present findings corrobotate with Sadler *et al.* (2000), who reported that Atlantic salmon (*Salmo salar*) subjected to acute handling stress had higher cortisol levels (75.5 µg/dl and 74.3 µg/dl) than unstressed ones (24.6 µg/dl and 32.6 µg/dl). According to Giulia *et al.* (2008) the cortisol level increased from 200 µg/dl to 600 µg/dl in *Dicentrachus labrax* due to short term acute stress, which was also in accordance with the present findings. Hosoya *et al.* (2006), recorded noticeable variation in serum cortisol in juvenile haddock (*Melanogrammus aeglefinus*), and reported an increase in cortisol level from 5 µg/dl to 45 µg/dl when they were exposed to acute handling stress and 37 µg/dl to 56 µg/dl when subjected to 1 week handing every day. When rainbow trout (*Oncorhynchus mykiss*) was subjected to acute stress serum cortisol level increased significantly from 1.5 µg/dl to 5.5 µg/dl (Kubuley *et al.,* 2002). Similarly, the mean cortisol level in jundia (*Rhamdia quelen*) was

significantly higher in stressed fish (180 µg/dl) than control (35 µg/dl) (Leonardo *et al.,* 2003). On contrary to the present findings and earlier reports, Svoboda *et al.* (1999) observed decrease in cortisol form 650 mmol/l (23.4 µg/dl) to 630 mmol/l (22.68 µg/dl) in common carp (*Cyprinus carpio*) subjected to short term acute handling (5 min).

5.2.9.3. Transportation stress

The serum cortisol changed with transportation procedures as well as density of packing in comparison to undisturbed fishes. The mean cortisol level in the unstressed fish (BD) was relatively lower (20.00±2.00 µg/dl) and increased as the transportation procedure begun. The value was 29.00±3.00 µg/dl before transportation (BT). Immediately after transportation it further augmented to 52.00±2.00 µg/dl, 56.00±1.00 µg/dl, and 56.00±0.00 µg/dl in fish stocked at 500 g/bag (AT1), 1.0 kg /bag (AT2) and 1.5 kg/bag (AT3), respectively. The serum cortisol showed significant differences ($P< 0.05$) between BT and ATs (of all the packing densities). Dobsikova *et al.* (2009) reported increased cortisol levels in common carp (*Cyprinus carpio*) subjected to transportation stress. The cortisol level increased from 258.57 ng/ml (25.85 µg/dl) to 283.38 ng/ml (28.33 µg/dl) and 301.94 ng/ml (30.19 µg/dl) when subjected to 7h and 12h transportation stress, respectively. According to Aberu *et al.* (2008) cortisol level increased from 40 ng/ml (4 µg/dl) to 120 ng/ml (12 µg/dl) in juvenile matrinxa (*Brycon amazonicus*) transported for 4h. A significant increase in cortisol level was also found in juvenile tambaquii (*Colossoma macropomum*) from 80 ng/ml (8.0 µg/dl) (before disturbance) to 125 ng/ml (12.5 µg/dl) before transportation and further it increased to 140 ng/ml, (14.0 µg/dl) 200 ng/ml, (20.0 µg/dl) 180 ng/ml (18.0 µg/dl) and 220 ng /ml (22.0 µg/dl) immediately after transportation and then decreased to 60 ng/ml, (6.0 µg/dl) 140 ng/ml, (14.0 µg/dl), 110 ng/ml (11.0 µg/dl) and 135 ng/ml (13.5 µg/dl) after 24 hrs of in case of 78 kg/cubic meter, 156 kg/cubic meter, 234 kg/cubic meter and 312 kg/cubic meter packing densities, respectively (Carlos *et al.,* 2003). Contrary to the above findings, Dobsikova *et al.* (2009) recorded decrease in cortisol level from 213.3 ng/ml (21.33 µg/dl) to 206.6ng/ml (20.6 µg/dl) and 201.6ng/ml (20.16 µg/dl) in common carp when subjected to 7h and 12h transportation stress.

5.2.9.4. Experimentally challenged bacterial infection stress

The mean cortisol content of *Labeo rohita* control (initial) was 19.00±1.00 µg/dl. In 6h post-*Aeromonas hydrophila* challenged fish it increased to 30.00±2.00 µg/dl. However, the 7 days post-challenged fish recorded a decrease in cortisol to 24.00±1.00 µg/dl. Likewise, the mean cortisol content obtained were 25.00±1.00 µg/dl and 23.00±2.00 µg/dl after 6h and 7 days, respectively in experimentally challenged rohu with *Edwardsiella tarda* at sublethal dose. There existed significant difference ($P<0.05$) between control and *Aeromonas hydrophila* infected fish (after 6h). The difference between the cortisol levels of control and *Edwardsiella* infected rohu was however, insignificant. Cortisol is commonly used as an indicator of the degree of stress experienced by fish (Barton and Iwama, 1991; Wendelaar Bonga, 1997). When fish are exposed to a stressor, the physiological stress response is initiated by the recognition of a real or perceived threat by the central nervous system (CNS). The sympathetic nerve fibers, which innervate the chromaffin cells, stimulate the release of catecholamine via cholinergic receptors (Reid *et al.*, 1996, 1998). The chromaffin tissue (adrenal medulla homologue) is located mainly in the anterior region of the kidney in teleostean fishes (Reid *et al.*, 1998). As catecholamines, predominantly epinephrine in teleostean fishes, are stored in the chromaffin cells, their release is rapid and the circulating levels of these hormones and increase immediately with stress (Mazeaud *et al.*, 1977; Randall and Perry, 1992; Reid *et al.*, 1998). The release of cortisol in fishes is delayed relative to catecholamine release. The pathway for cortisol release begins in the hypothalamic-pituitary-interrenal (HPI) axis with the release of corticotrophin-releasing hormone (CRH), or factor (CRF), chiefly from the hypothalamus in the brain, which stimulates the corticotrophic cells of the anterior pituitary to secrete adrenocorticotropic (ACTH). Circulating ACTH, in turn, stimulates the interrenal cells (adrenal cortex homologue) embedded in the kidney to synthesize and release corticosteroids into circulation for distribution to target tissues. Cortisol is the principal corticosteroid in teleosts (Sangalang *et al.*, 1971; Idler and Tsiiyuki, 1958; Hanson and Fleming, 1979; Barton *et al.*, 1998). This might be the reason for elevation in cortisol under stressed conditions.

CHAPTER-6

Summary and Conclusion

6. SUMMARY AND CONCLUSION

Fisheries sector plays a vital role in Indian economy through substantial foreign exchange earnings, employment generation and ensuring nutritional and food security. Aquaculture is the world's fastest growing food production system, increasing at a rate of eight percent annually. Rohu, *Labeo rohita* (Hamilton, 1822) is the most important among the three Indian major carp species used in polyculture systems. It is widely cultured throughout India owing to its high commercial value, good growth rate, consumer preference and acceptability to artificial diets. Rapid growth of aquaculture also resulted in increased occurrence of problems leading to diseases and economic losses. Fish under aquaculture conditions are invariably subjected to physical, chemical and biological stressors. Exposure of fish to such stressors can elicit physiological changes. Stress mitigation is one of the most challenging tasks in aquaculture and is the most promising areas of research. Haematology and biochemistry play an important role in monitoring not only the stress and health status of fish, but also serves as diagnosis of metabolic disturbance and structural and functional status of the body. However, detailed study on the effects of acute stressors on haematological and biochemical paramaters including metabolic enzymes of carps, especially rohu (*Labeo rohita*) is scanty. Therefore, the present study is carried out to assess the effect of acute physical and biological stressors on the haematological and biochemical responses in rohu *Labeo rohita* (Hamilton, 1822). The results obtained are summarized below.

The blood parameters at different stressors revealed a specific trend of significant increase or decrease with the severity of stressor. However, the lowest values were mostly observed in the control fishes.

The mean total erythrocyte count (TEC) showed increasing trend with stress and rate of increment was more in acute single handling stress where highest value was found in ASHS3 (15 min acute handling) ($2.00\pm0.00 \times 10^6$ no/ml) and the lowest value was noticed in control ($1.15\pm0.05 \times 10^6$ no/ml).

Total leukocyte count (TLC) exhibited a sharp increasing trend with stress and rise was more in acute single handling stress where maximum value was found in ASHS3 (15 min acute single handling) ($99.00\pm2.00\times10^3$ no/ml). In bacteriological stress total TLC value did not differ much in comparison to other stressor factor.

Haemoglobin and haematocrit contents did not reveal a specific trend of significant increase or decrease with the rise in stress level. The Hb content was maximum (5.20±0.20

g/dl) in fish subjected to acute single handling stress, however, the peak Ht value (41.20±0.03 %) was observed in AT3 (in fish packed at 1.5 kg/bag after transportation).

The total protein was the only parameter that showed a declined trend. The maximum protein content (8.28±0.28 g/dl) was observed in unstressed fish where as minimum value was noticed in ALHS3 (1.81±0.07 g/dl)

Serum glucose and serum cortisol increased with the severity of stress subjected to different stressors. The peak glucose was observed in fish subjected to acute long term handling stress in higher stocking density (113.5±1.5 mg/dl). The cortisol content was maximum (60.00±2.00 µg/dl) in fish subjected at acute long term handling stress, whereas the cortisol concentration was lowest in the control fish (19.00±1.00 µg/dl).

Serum triglycerides and serum urea did not display a specific trend of significant increase or decrease with the rise in stress. However, the peak triglycerides value was noticed in bacteriological stress. Highest level of triglycerides (81.50±1.50 mg/dl) and urea (7.00±1.00 mg/dl) were found after 6 hours of *Aeromonas hydrophilla* inoculation where as the lowest triglycerides (24.00 ±2.00 mg/dl) as well as lowest urea (3.95±0.50 mg/dl) value was found in control fish.

Serum creatinine level also did not illustrate significant difference with any stress though highest level (0.65±0.02 mg/dl) was recorded in case of transportation stress at highest stocking density (AT3) and the lowest value was recorded in (0.28±0.02 mg/dl).

All the serum enzymes flaunted a drastic increase trend with different stressors. Highest level of alanine aminotransferase (33±3 IU/L) and aspartate aminotransferase (60.00 ±2.00 IU/L) were found in ALHS3 (15 min/day) acute long term handling stress, where as the lowest alanine aminotransferase (10±1 IU/L) and aspertate aminotransferase, (37±2.00 IU/L) were found in the control fish. Highest level of lactate dehydrogenase (192.00±2.00 IU/L) was found AT3 (in fish packed at 1.5 kg/bag after transportation) as against lowest level of 71.00±6.00 IU/L found in unstressed fish.

Although the blood parameters varied differently at different stress factor but in a general observation of each blood parameter showed a sharp increase with severity of stress factor. And higher increasing trend was observed at acute single handling stress as well as *Aeromonas hydrophila* inoculated fish.

Packing was found to have a profound effect on water quality. Temperature has increased after transportation while dissolved oxygen declined in all the packing densities with profound decrease in the bag containing 1.5 kg/bag.

Haematological and biochemical parameters have been acknowledged as valuable tools for monitoring fish stress and health. The ranges of normal values of key biochemical parameters are still undefined for different species in different aquaculture conditions. The results of this research provide the knowledge of the characteristics of haematological parameters of rohu, *Labeo rohita*. Acute single handling stress is found to be more severe than that of acute long term handling considering the sensitivity of parameters. Based on the results of the present study from physiological point of view an appropriate packing density for fish transportation for 2-3 h duration should be 1 kg/bag (1 kg/7.5 l of water) approximately as against 1.5 kg/bag (1.5 kg/ 7.5 l of water) as in practice. *Aeromonas hydrophila* infection caused more severe effect on the haematological and biochemical responses of *Labeo rohita* than *Edwardsiella tarda* infection. Although many of the parameters indicated altered physiological conditions of fish, it can be concluded that all the parameters may not serve as good indicators of stress. Nevertheless, parameters such as serum glucose, total protein, alanine aminotransferase, aspartate aminotransferase and cortisol showed higher sensitivity to acute stressors evaluated in the present study. Further studies are, therefore, recommended under different stress conditions to formulate suitable stress mitigating strategy.

CHAPTER-7

References

8. REFERENCES

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CHAPTER-8

Annexure

ANNEXURE – I

Composition of Dacie's fluid or RBC diluting fluid

Chemicals	Proportion
Formaldehyde	1.00 ml
Trisodium citrate	3.13 g
Brilliant cresyl blue	0.10 g
Distilled water	100 ml

ANNEXURE – II

Composition of Shaw's W.B.C. diluting fluid

Chemical used	Proportion
Glacial acetic acid	3.00 ml
Gention violet	10.00 mg
Distilled water	97.00 ml