EXPRESSION OF PLASMA GLUCOSE TRANSPORTER PROTEIN 1 (GLUT 1) INDUCED BY CHRONIC STRESS IN NILE TILAPIA (*Oreochromis niloticus* Linnaeus 1758) REARED AT HIGH STOCKING DENSITY

BY

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ABSTRACT

High stocking density (HSD) has been found suitable to overcome the problem of land shortage, and it has the desirable effect of increasing fish production hence a potential profit maker. However, HSD is a chronic stressor that reduces growth rates; increases immunosuppression and susceptibility to diseases; and may lead to stock loss. HSD is likely to lower profitability of fish farming if not implemented properly. Stress elevates plasma cortisol levels, which in turn promotes carbohydrate metabolism leading to high blood glucose levels. Glucose, the cellular biofuel, is transported to various tissues by glucose transporter protein (GLUTs) in order to maintain normalcy. GLUT 1 is located in erythrocyte membrane and has high preference for glucose, and is thus responsible for transport of glucose in Erythrocytes. However, knowledge about how glucose relates to GLUT 1 protein in fish subjected to HSD is still unclear. Therefore, this study examined the effect of HSD on the plasma levels of GLUT 1 protein in Nile tilapia. Fish were reared for 28 days at 1.5 kg/ m^3 and 4.5 kg/m^3 for low stocking density (LSD) and HSD respectively. Cortisol levels were measured by competitive ELISA. Blood glucose level, haemoglobin concentration (Hb), and haematocrit (Hct) were also measured. Erythrocytes count and differential leukocyte count were determined by haemocytometer. GLUT 1 levels were determined by SDS-PAGE and Western blot. HSD fish had high cortisol level, glucose level, Hb, Hct, erthrocyte count, neutrophils, and Monocytes ($p < 0.05$). HSD individuals had lower lymphocyte numbers compared to LSD individuals ($p < 0.05$). Significantly higher GLUT 1 levels were detected in HSD individuals ($\bar{x}$±SEM = 2.0±0.22) compared to LSD individuals ($\bar{x}$±SEM = 0.25±0.06), $p < 0.05$. This data point to the fact that HSD induces chronic stress that interferes with the normal physiology of fish. The result from this study also shows that HSD heightens expression of GLUT 1 protein in blood, and as such the level of GLUT 1 can be used as a stress indicator in fish farming.

CHAPTER ONE: INTRODUCTION

1.1 Background information

Nile tilapia, *Oreochromis niloticus* (Linnaeus 1758), is one of the most important and commonly farmed tilapia fish in the world owing to its ability to reach early maturity, easy acclimation in a wide range of environmental conditions, ready acceptance of artificial feed, and has a high reproductive capacity (Fujimura & Okada, 2007; Fitzsimmons, 2004; Peschiri and Yakupitiyage, 2005). Nile tilapia is cultured either for subsistence or commercial needs where the great majority of the culture practices are semi-intensive (Liti *et al.*, 2005). However, overpopulation of the species in confined ponds leads to territoriality and competition for food subsequently resulting in production of stunted fish population of poor market value (Fitzsimmons, 2000; 2004). Various methods of population control have been applied such as culture in cages, culture with predators, intermittent harvesting, induction of sterility and mono sex culture of male tilapia.

Nile tilapia is also an important model in scientific research studies worldwide. Lee *et al.* (2003) reported that sex-differentiation in fish is controlled by specific sex-determining genes, and that interaction between the genome and variable environmental and internal factors may determine sex. Sex-determination in Nile tilapia occurs through genetic mechanism usually in one of two forms: heterogametic male (XY) or heterogametic female (WZ) (Donaldson, 2000). However Muller-Belecke and Horstgen-Schwark (1995) reported that two or more sex determining factors might override the XX–XY mechanism in Nile tilapia. Heat induced sex-determination in *O. niloticus* is regulated by *Abur36* locus on sex-linked gene which interact with temperature to give

male biased sex ratio (Ang'ienda *et al.*, 2010). Temperature dependent sex reversal is the most effective and environmentally friendly in terms of production and adoptability to local farmers for seed production in facilitating mono sex culture of all-male *O. niloticus* (Ang'ienda *et al.*, 2010).

1.1.1 Fish intensification practices and stress

Nile tilapia culture is widely practiced, either at extensive or semi-intensive levels, in many parts of the world (Salama *et al.*, 2006). In Africa, aquaculture is almost entirely for subsistence, with little surplus production being sold in the rural market. Most fish farming in sub-Saharan Africa is for subsistence, undertaken by rural folk in small ponds as a secondary activity to agriculture. Thus, Africa contributes only 0.2% towards the world aquaculture production, and this is from only a handful of countries, notably Kenya, Madagascar, Nigeria, Zambia and South Africa, though production is expected to rise steadily (Omondi *et al.*, 2001). The semi-intensive culture of tilapia is particularly ideal in Africa because it provides a wide variety of options in management and capital investments. Management strategies in the lower levels of intensification involve the use of fertilized-ponds, manipulation of stocking density, supplemental feeding, and water quality management. Proper implementation of these management practices will increase productivity (Salama *et al.*, 2006). For instance, fish yields from fertilized-ponds were found to be higher than those from unfertilized ponds (Green, 1992). Higher fish yield was achieved by use of supplemental feeding and fertilized-pond techniques combination, which resulted in rapid growth of tilapia and large size within a shorter time compared to when fertilized-pond technique was used alone (Green, 1992; Diana, 1997; Brown *et al.*, 2000). Fitzsimmons (1997) reported that rapid growth for whole populations can only be achieved under favorable conditions of water quality.

Maximizing fish production through intensive culture has been shown to improve profitability of fish farms, and is also suitable in alleviating the problem of land shortage (Abdel-Tawwab *et al.*, 2005; Chakraborty *et al.*, 2010). Many fish farmers stock at high density expecting high returns, but in doing so, they unknowingly subject their fishes to conditions that impair fish's welfare (Lembo and Zupa, 2008). High stocking density predisposes fish to stress and diseases, which in turn, affect farm productivity (Schreck, 1996). It has been shown that poor water quality and alteration of behavior (increased competition, aggression, physical injury and cannibalism) are responsible for detrimental phenomena associated with high stocking density namely: decreased growth; poor nutritional status; increased food conversion rate; fin erosion; disruption of reproductive capacities; increase to disease susceptibility; and mortality (Jobling and Reinsnes, 1986; Schreck and Bradford, 1990; Al-Harbi and Siddiqui, 2000; Ellis *et al.*, 2001).

In fish farming there are several potential stressors other than HSD. Handling, transport, capture, confinement, and overcrowding are a common occurrence in fish husbandry that causes stress. Inappropriate composition and timing of feeding regimes could cause the impairment of fish welfare. Feed composition is important for preserving welfare (Lembo and Zupa, 2008). Diets lacking in vital micronutrients impair welfare, causing morphological abnormalities, poor immune function, abnormal behavior and slow growth (Huntingford *et al.*, 2006). Feed distribution in a small area could generate competition and increased aggression among fish that in turn could lead to growth variations reinforcing dominance hierarchies (Caruso and Lazard, 1999). Aggression and cannibalism cause injury in farmed fish, especially when competition for food is strong (Greaves *et al.*, 2001). Low oxygen levels and exposure to pollutants may predispose fish to disorders associated with oxidative stress (Bainy *et al.*, 1996).

1.1.2 The stress responses

Upon detection of a stressor by the central nervous system (CNS) of fish, catecholamines (Epinephrine and Nor-epinephrine) are released by chromaffin tissue (adrenal medulla homologue). These hormones are produced beforehand and stored in secretory vesicles (chromaffin cells) and, therefore, their release occurs rapidly within seconds after detection of a stressor (Barton, 2002; Romero and Butler, 2007). They activate beneficial responses such as increasing alertness and providing energy to muscles during an acute emergency. However, the half-life of catecholamines is transient and therefore it is not a suitable stress indicator because it is not easy to measure its levels.

The release of cortisol in teleost fishes is delayed relative to catecholamine release. The pathway for cortisol release begins in the Hypothalamo-Pituitary-Interrenal (HPI) axis with the release of corticotrophic-releasing hormone (CRH), chiefly from the hypothalamus in the brain, which stimulates the corticotrophic cells of the anterior pituitary to secrete adrenocorticotrophic hormone (ACTH). Circulating ACTH, in turn, stimulates the Interrenal cells to synthesize and release corticosteroids into circulation for distribution to target tissues. Cortisol is the principle corticosteroid in teleost fish ((Pickering and Pottinger, 1989; Pickering, 1993; Barton, 2002). Cortisol synthesis and release from Interrenal cells has a lag time of several minutes, and, therefore, proper sampling protocol can allow measurement of resting levels of this hormone in fish (Barton, 2002). As a result, the circulating level of cortisol is commonly used as an indicator of the degree of stress experienced by fish (Barton and Iwama, 1991; Wendelaar Bonga, 1997; Mommsen *et al.*, 1999). Mean basal Cortisol levels' ranging from 5 – 60 ng/ml is considered normal for *O. niloticus* (Auperin *et al.*, 1997; Barreto and Volpato , 2001; 2006). Control of cortisol release is through negative feedback of the hormone at all levels of the HPI

axis (Wendelaar Bonga, 1997). An elevation of plasma cortisol induces a wide variety of secondary physiological responses (Costas *et al.*, 2008), including regulation of calcium absorption, blood pressure maintenance, anti-inflammatory function, gluconeogenesis, gastric acid and pepsin secretions, and immune function (Fig. 1.1). Other physiological responses of fish to stressors include changes in plasma and tissue ions and metabolite levels, haematological parameters (hemoglobin, haematocrit, erythrocytes and leukogramme), and cellular responses such as elevation of heat shock proteins (Mommsen *et al.*, 1999; Barton, 2002).

In aquaculture research, there is a common agreement among researchers that changes in haematological parameters mentioned earlier are attributable to chronic stress (Schreck, 1990; Barton, 2002). Therefore, there has been a greater understanding of the need to establish reference haematological and biochemical values in fish in order to assess health status and the subsequent diagnosis of disease. Bittencourt et al (2003) and Maule et al (2007) established reference ranges of various haematological parameters of a healthy Nile tilapia, which would provide a reference for fish farmers evaluating the health of cultured tilapia in a variety of aquaculture systems (Table 4.1, selected biochemical and haematological parameters). Changes in the levels of haematological parameters away from their corresponding reference ranges is an indication of physiological disturbances experienced by fish during stress (Witeska, 2005)

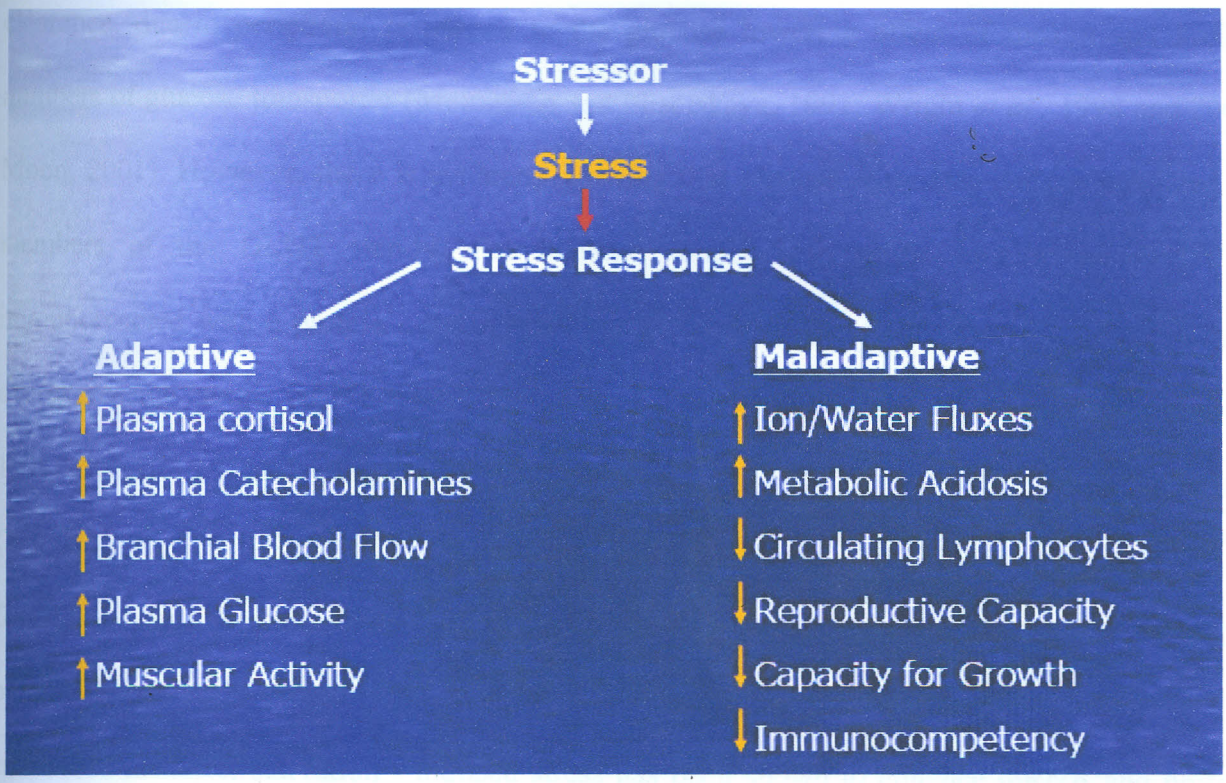

Figure 1.1: Stress and stress responses (Adapted from Mustafa, 2008)

1.1.3 Effects of stress on glucose metabolism

Cortisol affects the metabolism of carbohydrates, protein and lipid. Generally Cortisol is hyperglycemic, and mediates increased liver metabolic capacity such as glycogenolysis, glycolysis and gluconeogenesis; thereby elevating blood glucose levels [Fig. 1.1] that produces a burst of energy which prepares the fish for an emergency situation (Pickering and Pottinger, 1989; Pickering, 1993; Mommsen *et al.*,, 1999; Wiseman *et al.*, 2007). Thus, high blood levels of glucose can be used as secondary indicator of stress. The resultant glucose is transported into target cells and tissues of the host (Wood and Trayhurn, 2003). The movement of glucose across cell membranes is mediated through a group of facultative glucose transporters known as glucose transporter proteins (GLUTs).

Teleost fish are known to be glucose intolerant species with very slow rates of plasma glucose clearance, due to peripheral resistance to the glucose lowering effect of insulin and limited numbers of glucose transporter (Mommsen and Plisetskaya, 1991; Wright *et al.,* 1998 and 2000; Moon, 2001). However, there is evidence showing that glucose entry into cells is mediated by members of the GLUT family. For instance, antibodies against mammalian GLUT 1 immunoreacted with a protein only in heart and brain of tilapia; and transgenic trout embryos overexpressing human GLUT 1 showed increased glucose uptake and metabolism (Wright *et al,* 1998). GLUT 1 is ubiquitously expressed in the brain, heart, gills, muscle and erythrocytes' (Wright *et al.*, 1998; Klepper, *et al.*, 1999). However, more abundant expression of GLUT 1 is found in erythrocytes and brain. GLUT 1 is a stress-inducible protein of the glucose-regulated proteins superfamily (Wertheimer *et al.*, 1991) of which the expression level is modulated by stress. For instance, starvation - induced stress has been shown to enhance mRNA and protein levels of GLUT 1 in muscle of diabetic rat (Kahn *et al.*, 1991). Hrytsenko *et al* (2010) observed that acute glucose injection of glucose up-regulated the mRNA GLUT 1 levels in white muscle of fish. It is against this background that the present study attempts to answer these questions: a) does HSD – induced stress modulates the levels of GLUT 1 protein in erythrocytes of Nile tilapia subjected to high stocking density? b) Does GLUT 1 play a role in clearance of endogenous glucose from blood of fish subjected to HSD? Therefore, the aim of this study was to determine if high stocking density, a known chronic stressor, had effect on the levels of GLUT 1 protein in Nile tilapia during long episodes of stress.

1.2 Rationale

In aquaculture, high stocking density is used to maximize water usage with the sole purpose of increasing productivity. However, high stocking density is known to produce chronic stress by affecting water quality. Under stress conditions, the fish usually mobilize energy reserves towards activities that restore homeostasis instead of investing the energy in vital physiological functions such as growth and immunity, and ultimately the fish suffers growth suppression and mortality. Stress has the potential of lowering profitability of fish farming. The innate stress response present in fish is such that increase in cortisol level will result in elevation of blood glucose level. The GLUTs transport the resulting glucose across cell membranes and, therefore, it is important for the control of glucose homeostasis. It has been shown that impaired glucose transport (which may be due to non-functional GLUT 1 or its absence) across brain-barriers causes infantile seizures, developmental delay and acquired microcephaly (Klepper *et al.*, 1999). GLUT 1 protein has a wide tissue distribution, however, its locality in erythrocytes and its exclusive predilection for glucose makes it the right candidate for glucose transport in erythrocytes. It has been reported that stress modulates the mRNA and protein levels of GLUT 1 (Kahn *et al.*, 1991). To date no study has examined the effect of HSD (a chronic stressor) on GLUT 1 levels, and the role of GLUT 1 in glucose metabolism during stress episodes in Nile tilapia. Therefore, this study investigated the effect of high stocking density on the levels of GLUT 1 protein in Nile tilapia, *O. niloticus*.

1.3 Hypothesis

1.3.1 Null hypothesis

1. High stocking density does not affect levels of expression of GLUT 1 in Nile tilapia.

2. High stocking density-induced stress does not affect levels of plasma cortisol, blood glucose, haemoglobin, haematocrit, erythrocyte count, and differential leukocyte count in fish

1.4 Objectives

To determine the effect of stocking density on the expression of glucose transporter protein 1 (GLUT 1) protein in Nile tilapia.

1.4.1 Specific objectives

1. To measure levels of GLUT 1 protein in *O. niloticus* reared at high and low stocking density.

2. To determine the presence of stress in *O. niloticus* reared at high and low stocking density.

3. To measure levels of haematological indices (haemoglobin, haematocrit, erythrocyte count and differential leukocyte count) in high and low stocking densities fish.

CHAPTER TWO: LITERATURE REVIEW

2.1. Biology and ecology of Nile tilapia

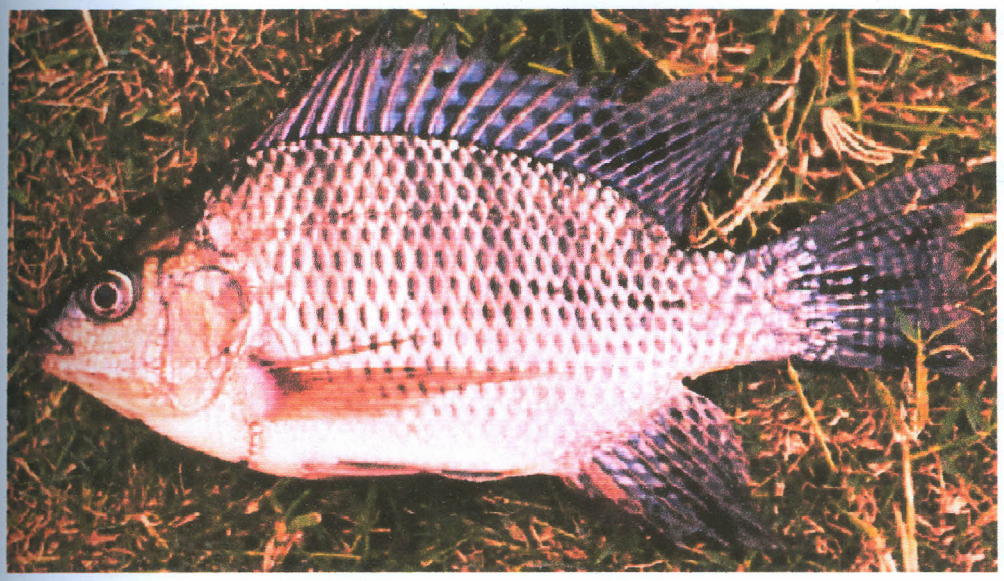

Figure 2.1. Nile tilapia (*Oreochromis niloticus* L. 1758).

Nile tilapia, *Oreochromis niloticus*, belongs to class Osteichthyes, order Perciformes, family Cichlidae, and genus *Oreochromis*. Nile tilapia has a compressed body (Fig 2.1) and cycloid scales. It has gill arch with 27 to 33 gillrakers, and has interrupted lateral line. Its dorsal fin is spinous and continuous, with 16 to 17 spines and 11 to 15 soft rays. Anal fin has 3 spines and 10 to 11 rays. Caudal fin is truncated. During spawning season, pectoral, dorsal and caudal fins becoming reddish in colour (Wooton, 1990).

Nile tilapia is a tropical species that prefers to live in shallow water with lower and upper lethal temperatures of 8-12 °C and 42 °C, respectively, while the preferred temperature ranges from 23 to 27 °C (Al-Harbi and Siddiqui, 2000). It feeds on phytoplankton, periphyton, aquatic plants, small invertebrates, benthic fauna, detritus and bacterial films associated with detritus. It is also a filter feeder and it entraps suspended particles such as phytoplankton and bacteria on mucous buccal cavity (Popma and Masser, 1999). Sexual maturity in ponds is reached at an age of 5-6 months (Wooton, 1990). Spawning begins when the water temperature reaches 24 °C. The breeding process starts when the male establishes a territory, digs a craterlike spawning nest and guards his territory. The male will fertilize the eggs laid in the nest by the ripe female. After fertilization the female then collects the eggs into its mouth and moves away from the nest (Keenleyside, 1991). The female incubates the eggs in its mouth and broods the fry after hatching until the yolk sac is absorbed. Incubating and brooding is accomplished in 1 to 2 weeks, depending on temperature (Wooton, 1990; Beveridge and McAndrew, 2000). Being a maternal mouth brooder, the number of eggs per spawn is small in comparison with most other pond fishes. Egg number is proportional to the body weight of the female. For instance a 100 g female will produce about 100 eggs per spawn, while a female weighing 600-1 000 g can produce 1 000 to 1 500 eggs (Pullin and Lowe-McConnell, 1982; Beveridge and McAndrew, 2000). The male remains in his territory, guarding the nest, and is able to fertilize eggs from a succession of females. While the female is brooding, she eats little or nothing. Nile tilapia can live longer than 10 years and reach a weight exceeding 5 kg (Pullin and Lowe-McConnell, 1982; Beveridge and McAndrew, 2000).

2.2 Water quality parameters

Nile tilapia is more tolerant than most commonly farmed freshwater fish to high water temperature, low dissolved oxygen, and high ammonia concentrations (Bardach, 1972). Tilapia survive routine dawn dissolved oxygen (DO) concentrations of less than 0.3 mg/L, considerably below the tolerance limits for most other cultured fish. Ex-situ studies have shown that Nile tilapia grew better when aerators were used to prevent morning DO concentrations from falling below 0.7 to 0.8 mg/L (Al-Harbi and Siddiqui, 2000). Although tilapia can survive acute low DO concentrations for several hours, tilapia ponds should be managed to maintain DO concentrations above 1 mg/L. Metabolism, growth and disease resistance are depressed when DO falls below this level for prolonged periods (Wooton, 1990). Tilapia can survive in pH ranging from 5 to 10 but do best in a pH range of 6 to 9 (Bardach, 1972; Wooton, 1990). Experiments to establish temperature tolerance have shown that the species *O. niloticus* is able to tolerate temperatures ranging from 8-42°C (Wooton, 1990). Temperature preference depends on size, young *O. niloticus* being more tolerant to higher and lower temperatures than adults (Wooton, 1990).

Even though massive mortality occurs within a few days when Nile tilapia fish are suddenly transferred to water with un-ionized ammonia concentrations greater than 2 mg/l, gradual acclimation to sublethal levels increases survival rates by 50% at un-ionized ammonia concentrations as high as 3 mg/l. Prolonged exposure (5-7 weeks) to un-ionized ammonia concentration greater than 1 mg/L causes deaths, especially among fry and juveniles in water with low DO concentration. Un-ionized ammonia begins to depress food consumption at concentrations as low as 0.08mg/L (Keenleyside, 1991).

Nitrite poisons fish by binding the haemoglobin in the blood preventing it from carrying oxygen, thus suffocating the fish. Tilapia fish are more tolerant of nitrite than many cultured freshwater fish. In one study, nitrite concentration of 89 mg/L killed Nile tilapia when DO concentration was as high as 6 mg/L and chloride concentration was as low as 22 mg/L (Keenleyside, 1991). In general, for freshwater culture the nitrite concentration should not exceed 0.2mg/l (Al-Harbi and Siddiqui, 2000). As a safeguard against nitrite toxicity in recirculating systems, chloride concentrations should always be maintained at 100 to 150 mg/L chloride.

2.3. Nile tilapia aquaculture in Kenya

Commercial production of tilapia is increasingly gaining expansion in many countries due to its suitability to variety of pond farming conditions, resistance to diseases, high survival and growth rate (Pullin *et al.*, 1991). In Kenya, development policy by the government has put a lot of emphasis on poverty eradication via increasing food production. The Government through the Department of Fisheries has been involved in programmes that promote aquaculture development through various aquaculture projects over the past few decades. This has resulted in the current impetus in the growth of aquaculture and small-scale fish operations. The end result is that now aquaculture contributes more than 1% of the total national production of protein, with Nile tilapia culture contributing at least 75% of fish produced through aquaculture (Kaliba *et al.*, 2007). However, the persistent demand for protein diet has led to expansion of Nile tilapia culture due to consumers' penchant for the 'aquaculture chicken'. In order to keep up with the ever rising demand for fish protein, fish farmers have engaged in practices that are intended to increase fish production. These practices include Polyculture of Nile tilapia and catfish, Mixed-sex Nile tilapia culture, and increasing stocking density among others (Abou *et al.*, 2007; Kaliba

et al., 2007). The latter two practices have inherent shortcomings namely early maturity among females; prolific reproduction leading to stunted populations; and causation of chronic stress that eventually reduce growth rate, increases disease susceptibility and mortality rates (Costas *et al*, 2008; Montero *et al*, 1999). This may translates into huge losses to the fish farmer.

2.4 Stress responses

2.4.1 Effect of HSD on cortisol and glucose levels

In aquaculture, the term stocking density refers to total number of fish or fish biomass per unit volume of water in the culture system. It is considered to be one of the important factors that affect fish growth, feed utilization and gross fish yield (Chakraborty *et al.*, 2010). High fish stocking density has been reported to produce chronic stress (Montero *et al.*, 1999; Costas *et al.*, 2008). In Nile tilapia, stocking density equal to or greater than 4.5 Kg/m^3 is considered high given that it decreases growth (due to stress), in comparison to low density of 1.5 Kg/m^3 (Al-Jerian, 1998). The Hypothalamic- pituitary- Interrenal (HPI) axis in fish is often stimulated in response to most forms of stress (Pickering, 1993), leading to release of cortisol hormone. As a result, the circulating level of cortisol is commonly used as an indicator of the degree of stress experienced by fish (Barton and Iwama, 1991; Wendelaar Bonga, 1997). Mean basal Cortisol levels' ranging from 5 – 60 ng/ml is considered normal for *O. niloticus* (Auperin *et al.*, 1997; Barreto and volpato, 2001 & 2006). Elevation of plasma cortisol level induces a wide variety of secondary physiological responses in an attempt to compensate for the challenge imposed upon it, and thereby cope with the stress (Wendelaar Bonga 1997; Iwama, Afonso & Vijayan, 2004).

Under resting conditions, cortisol sustains normoglycemia and prevents arterial hypotension. In the stressed state, elevated cortisol activates central nervous system thus increasing blood glucose concentration and elevating mean blood pressure in order to cope with stress (Mommsen *et al.*, 1999). Stress is an energy-demanding process that increases metabolic rate and oxygen uptake in fish (Barton *et al.*, 1987). To cope with the increased energy demand, fish mobilize substrates to fuel cellular processes. Glucose is an important fuel for metabolism and certain tissues (brain, heart, blood cells, and gills) rely primarily on glucose (Mommsen *et al.*, 1999) in order to execute their vital roles.

Elevated cortisol during stress plays a role in the immediate production of glucose by increasing glycogenolysis (via alteration in the phosphorylation-dephosphorylation status of glycogen phosphorylase or epinephrine/glucagon-mediated glycogenolysis) and gluconeogenesis processes (Mommsen et al., 1999). Thus, glucose is synthesised and its blood level is elevated as a result of these metabolic processes. Once glucose is synthesized, it is transported to the different cells and tissues of the host. The movement of glucose across cell membranes is mediated through a group of facultative glucose transporter proteins (GLUTs).

2.5. Facultative glucose transporter proteins

Diffusion of monosaccharides across the plasma membrane into cells is a key stage in carbohydrate metabolism that is regulated and facilitated by a family of transmembrane glycoproteins. Currently, 13 members of the facultative glucose transporter family (GLUT 1-13) have been identified in mammals (Joost and Thorens, 2001; Zhao and Keating, 2007). They share similarity in structure but differ in tissue or cell specificity, selectivity to substrates, and kinetics characteristics (Mueckler, 1994). Acting cooperatively, GLUTs supply cells with an

essential source of energy, and also maintain blood glucose levels within the normal limits. All known GLUTs are composed of a single polypeptide chain and contain 12 transmembrane spanning amphipathic helices with the amino and the carboxy terminus residing on the cytoplasmic cell surface (Mueckler, 1994; Barrett *et al.,* 1999; Joost and Thorens, 2001). GLUT proteins transport glucose and related sugars, by exposing a single substrate binding site toward either the outside or the inside of the cell. Binding of glucose to one site provokes a conformational change associated with transport, and releases glucose to the other side of the membrane (Fig. 2.2). The mechanism by which the glucose transport is regulated may involve an increase in the content of glucose transporters in the plasma membrane primarily via translocation of glucose transporters from an intracellular pool to the plasma membrane and by changes in the turnover rate of the transporters (Lund *et al.*, 1995).

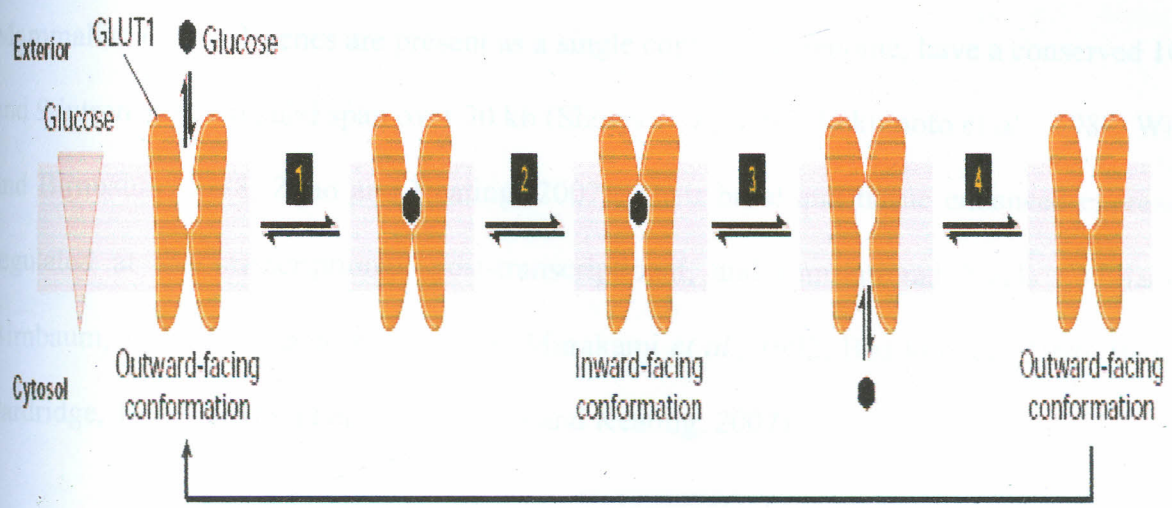

Figure 2.2. Model for uniport transport by GLUT 1 (Adapted from Lodish *et al* 2004)

Dysfunction of the GLUTs can lead to serious glucose homeostasis disorders; including type 1 and 2 diabetes (Li *et al.,* 1988; Mueckler, 1994; Zhao and Keating, 2007). Absence of GLUT 1 in the blood-brain barrier is known to cause infantile seizures, developmental delay and acquired microcephaly (Klepper *et al.*, 1999). Glucose transporter 1 (GLUT 1) is the most ubiquitous mammalian isoform, which is expressed at low levels in the majority of cell types and is responsible for the constitutive, non-insulin dependent glucose delivery into the cells (Mueckler, 1994). In adult mammals GLUT 1 concentrates in endothelial and epithelial cells of blood–tissue barriers (brain, placenta), indicating the essential role of this isoform in the transport of glucose across barrier tissues (Takata, 1996). In insulin sensitive cells, GLUT 1 interacts with the GLUT 4 isoform to cooperatively regulate glucose delivery to the cells under basal and insulin stimulated conditions (Marette *et al.,* 1992; Kraegen *et al.,* 1993; Fischer *et al.,* 1997). In human pancreatic islets GLUT 1 is a dominant isoform that, along with glucokinase, is involved in the glucosensing mechanism (De Vos *et al.,* 1995).

Mammalian GLUT 1 genes are present as a single copy in the genome, have a conserved 10 exon and 9 intron structure, and span over 30 kb (Shows *et al.,* 1987; Fukumoto *et al.,*, 1988; Williams and Birnbaum, 1988; Zhao and Keating, 2007). Their basal and tissue enhanced expression is regulated at the transcriptional, post-transcriptional, and translational levels (Williams and Birnbaum, 1988; Fukumoto *et al.,* 1988; Murakami *et al.,* 1992; Boado *et al.,* 1996; Boado and Pardridge, 1998; Griffin *et al.,* 2004; Zhao and Keating, 2007).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Experimental system

The present stock of Nile tilapia reared in ponds belonging to the Department of Zoology, Maseno University, were originally brought from Winam Gulf of Lake Victoria (appendix 1). Fish samples used in this study were the fifth generation of the parent stock. 30 fish (male and female) about 5 months old were obtained from ponds (where they were reared under tropical environmental conditions of photoperiod 12 hours of light and 12 hours of darkness, and temperature 23 - 27 ^{0}C) and then transferred to a 1000 L tank and let to acclimatize to their new environment for 7 days. Fish weighing 16.5 ± 3.1 grams were randomly stocked in 2 glass aquaria of 55 litres capacity at 1.5 Kg/m^3 and 4.5 Kg/m^3 representing LSD (control) and HSD (Experimental) respectively for 28 days (Al-Jerian, 1998; Al-Harbi and Siddiqui, 2000). Each aquarium was equipped with aerator pump for continuous aeration. Faeces and uneaten food residues were siphoned out of the tank together with about one half of the water volume of the aquarium each day and replaced with rain water. During acclimation and throughout the experimental period, the fish in each aquarium were fed twice daily with maize meal supplemented with 25% fish protein at 10 k per body weight (Ang'ienda *et al.*, 2010). Water quality was monitored throughout the study and recorded as given below; dissolved oxygen (5.0 ± 0.3 mg/l), pH (7.3 ± 0.2), temperature (26.3 ± 1.6 ^{0}C), nitrite (0.20 ± 0.02 mg/l) and total ammonia (0.04 ± 0.01 mg/l). The experiment was done in triplicate.

3.2 Sample collection and storage

At the end of the experiment, five fish from each aquarium were randomly sampled. To reduce stress, fish were quickly caught using hand-net, then anesthetized with 2-phenoxyethanol. Approximately 2 ml of blood was collected by cardiopuncture using a heparin–coated needle and syringe into EDTA-coated tubes. The blood samples were held on ice until all samples were collected. All sampling was completed within 5 minutes of fish removal from the aquarium. The following blood biochemical and haematological parameters (glucose, haemoglobin, haematocrit, erythrocyte count and differential leukocyte count) were determined from the whole blood. The remaining blood was centrifuged at 5000 rpm for 5 minutes to prepare plasma, which was then stored at -20^{0}C until assayed (Barreto and Volpato, 2006).

3.3 Blood parameters

Blood biochemical and haematological parameters such as glucose, haemoglobin concentration, haematocrit, red blood cell count and differential leukocytes count were determined according to the procedures based on Unified Methods for Haematological Examination of Fish (Svobodova et al., 1986).

3.3.1 Measurement of plasma cortisol by Enzyme-linked immunosorbent assay (ELISA)

Plasma cortisol levels were measured using a commercially available ELISA-kit (Cat # 402710, Neogen, Lexington, KY, U.S.A.) following protocols included with the kit. Plasma samples were diluted 1:10 fold in assay buffer for the extraction. The extracted samples (analyte) together with serially diluted cortisol standards were added in duplicates into wells of microplate pre-coated with cortisol antibodies. The plate was incubated with cortisol enzyme conjugate at room temperature for 1 hour, and then washed 4 times. The plate was again incubated with enzyme substrate at room temperature for 30 min. The plate was then read in a microplate reader at 650

nm (Appendix 2). A graph of optical densities (OD) verses concentrations of the standards was plotted (standard curve from table 7.1, Appendix 3). The concentrations of the samples (analyte) were then determined from standard curve (graph) and then multiplied by 10 (fig.7.0).

3.3.2. Blood glucose and Haemoglobin concentration

Blood glucose level was measured by handheld MD 300^{TM} glucose meter (MD Instruments Inc, USA). To measure blood glucose concentration a single drop of blood was placed on the blood strip and inserted in the meter and reading recorded to the nearest mg/dl according to the method established by Wells and Pankhurst, (1999). A single drop of blood was placed on strip and inserted in a Hemo-control machine (EKF Diagnostic, Germany) for measurement of Haemoglobin concentration following the method established by Wells and Pankhurst, (1999).

3.3.3. Haematocrit (Hct) concentration

Haematocrit (Hct) concentration was measured using standard volume microhaematocrit tubes. The blood was collected into each microhaematocrit tube to the 2 cm mark. One end of the tube was sealed using crit-o seal (sealing putty). The top head of the microhaematocrit centrifuge was closed tightly. Centrifugation was carried out for 5 minutes at 12,000 rpm. The tubes were then placed on microhaematocrit reader, and the percentage of packed cell volume recorded.

3.3.4. Erythrocyte count

Whole anticoagulated blood was diluted with the Natt and Herrick's solution at the rate of 1:200. The diluted blood was allowed to mix for 2 minutes before being discharged into the haemocytometer counting chamber. After charging the haemocytometer, the contents were allowed to settle for approximately 3 minutes. Enumeration of erythrocytes was done by counting the total number of erythrocytes in the four squares at the edge of each corner and

central square of the counting chamber. The total number of cells counted were multiplied by 10,000 to obtain the total erythrocyte count per/mm^3 (appendix 3).

3.3.5. Differential leukocyte count

The same dilution was used for both red and white cells, thus a total leukocyte count was obtained simultaneously from the same charged haemocytometer. The total leukocyte count was obtained by counting different leukocytes present in the nine large ruled squares of the haemocytometer.

Total leukocyte count was estimated through the formula:

Total WBC/µl - (total leukocytes in 9 squares + 10% of total WBC's) X 200

(See appendix 3 for more information).

3.4 Protein analyses by Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS - PAGE) and Western blot

3.4.1. SDS-PAGE: Plasma sample was mixed with 2X sample buffer in the ratio of 2:1, and warmed at 37°C for 30 minutes. 10 µl of both treated plasma samples and molecular weight (MW) marker [protein standard] were loaded into each well on the gel composed of 4% stacking and 12% resolving polyacrylamide inside glass plates clamped in vertical gel tank filled with running (electrode) buffer (appendix 4). Proteins were then resolved into fine bands on the polyacrylamide gel by SDS-PAGE technique at 150 V for 2 h following the method of Laemmli, (1970). After 2 hr, the running was stopped and the gel removed from glass plates (appendix 4). The gel was further subjected to western blot analysis (3.4.2) and then it was stained with Coomassie brilliant blue stain for visualization of the bands. The molecular weight (KDa) of protein of interest was then determined on the gel using the known weights of the protein ladder.

3.4.2. Western blot analysis: The resolved bands on the gel were transferred onto a 0.2-mm pore size nitrocellulose membrane at 17 V for 50 min with transfer buffer using a semidry transfer apparatus (Bio-Rad Trans-Blot, California, USA). Transfer membranes were blocked in 1× casein in Tween-20 Tris-buffered saline (TTBS; 17.4 mM Tris-HCl, 2.64 mM Tris Base, 0.5 M NaCl, and 0.05% Tween-20 [v/v]) for 1 h. Membranes were then rinsed once and soaked for 5 min in TTBS. The membranes were then incubated in a Mouse monoclonal IgG primary antibody for tilapia GLUT 1, in TTBS for 2 h at a dilution 1:5000 (Abcam, Cambridge, UK). After three, 5-min washes in TTBS, the membranes were incubated in Horse radish peroxidase (HRP) conjugated rabbit anti mouse IgG in TTBS secondary antibody for 1 h at a dilution 1:20000 (Abcam, Cambridge, UK). After three 5-min washes in TTBS and one 5-min wash in Tris buffered saline to remove Tween-20, the membranes were incubated for 1 min with enhanced chemiluminescence reagents Western blotting detecting system (Amersham, Chalfont, Bucks, UK). Exposure time to Kodak X-OMATAR film was 30 - 120 s (appendix 4). The film was scanned at resolution of 300-400dpi. The level of GLUT 1 was estimated against the constant level of a 51 KDa β-Tubulin (Abcam, Cambridge, UK) used as test standard by *ImageJ software* (Heidebrecht *et al.*, 2009) (appendix 5).

3.5 Statistical analyses

All the experiments were performed in triplicate. The results were analyzed using paired *t*-test of *GraphPad prism5* statistical software for Windows. The data were presented as mean ± SEM. Data were considered significantly different at the level of $p < 0.05$.

CHAPTER FOUR: RESULTS

4.1 Water quality parameters

The results for the water quality parameters measured are summarized in Table 4.0. These parameters were compared to the corresponding water parameters described as optimal conditions for Nile tilapia, and they were found to be close or within these established ranges (Al-Harbi and Siddiqui, 2000; Bardach, 1972; Keenleyside, 1991; Wooton, 1990). Based on the results obtained in this study, it was therefore concluded that the stress that affected the physiology of the fish emanated from stocking density treatment.

Table 4.0. Mean water parameters during stocking density experiments compared to optimal ranges of corresponding water parameters for Nile tilapia.

Parameter	Present experiment	Established (optimal) ranges
Temperature ^{0}C	26.3 ± 1.6	23 – 27*
pH	7.3 ± 0.2	6.0– 9.0**
Dissolved oxygen (mg/l)	5.0 ± 0.3	5.0 – 5.8*
Ammonia (mg/l)	0.04 ± 0.01	0.02 – 0.06***
Nitrite (mg/l)	0.20 ± 0.02	0.17 – 0.24***

* Al-Harbi and Siddiqui, 2000

**Wooton, 1990; Bardach, 1972

*** Keenleyside, 1991

4.2 Effect of HSD on haematological and biochemical characteristics of Nile tilapia

High stocking density individuals had higher cortisol level, glucose level, haematocrit, haemoglobin concentration, erythrocytes count, Neutrophils, and Monocytes than low stocking density individuals ($p<0.05$). However, high stocking density individuals had significantly lower lymphocyte numbers than low stocking density ($p<0.05$). The levels of all the blood parameters except for lymphocytes increased after stress induction by stocking at high density (Table 4.1; Fig. 4.1 – 4.8).

Table 4.1. Overall haematological and biochemical values of LSD and HSD individuals compared to the corresponding reference ranges described as normal for Tilapia fish in previous studies by Auperin et al. (1997), Bittencourt et al. (2003), Hrubec et al. (2000), and Mauel et al. (2007).

Parameters	LSD (Control) Mean ± SEM, (n = 15)	HSD (Experimental) Mean ± SEM, (n = 15)	*p* value	Reference values (Specific ranges)
Plasma cortisol level (ng/ml)	47.50 ± 4.55	72.10 ± 5.89	0.05	5 – 60*
Blood glucose level (mg/dL)	70.22 ± 2.55	136.00 ± 2.17	0.05	40.10 - 80.54**
Haemoglobin concentration (g/dL)	9.47 ± 0.10	9.86 ± 0.04	0.05	7.43 - 13.61**
Haematocrit %	31.79 ± 1.00	42.08 ± 1.50	0.05	23.40 – 40.30**
Erythrocytes count (10^6 mm^{-3})	2.47 ± 0.35	7.27 ± 0.48	0.05	1.35 - 15.21**
Monocytes (10^3 μl^{-1})	2.53 ± 0.51	5.07 ± 0.58	0.05	156 – 6,064*** (400 – 4,286)*[a]
Neutrophils (10^3 μl^{-1})	4.60 ± 0.77	10.30 ± 1.12	0.05	495 – 16,033*** (557 – 9,873)*[a]
Lymphocytes ($10^3 \mu l^{-1}$)	8.33 ± 0.80	2.47 ± 0.40	0.05	8,156 – 139, 610*** (9, 628 – 167,223)*[a]

* Values from studies by Auperin et al
** Values from studies by Bittencourt et al
*[a] Values from studies by Hrubec et al
*** Values from studies by Mauel et al

4.2.1 Effect of high stocking density on the levels of plasma cortisol

High stocking density individuals had significantly higher plasma cortisol level compared to low stocking density individuals ($p < 0.05$). Mean plasma cortisol level for HSD group ($\bar{x}$=72.1±5.89 ng/ml) was higher than the mean basal cortisol levels (5 – 60 ng/ml) described for Nile tilapia by Auperin et al. (1997); and Barreto and Volpato (2001; 2006), whereas the mean cortisol level for LSD ($\bar{x}$=47.05±4.55 ng/ml) individuals fell within the mean basal range (Fig. 4.1).

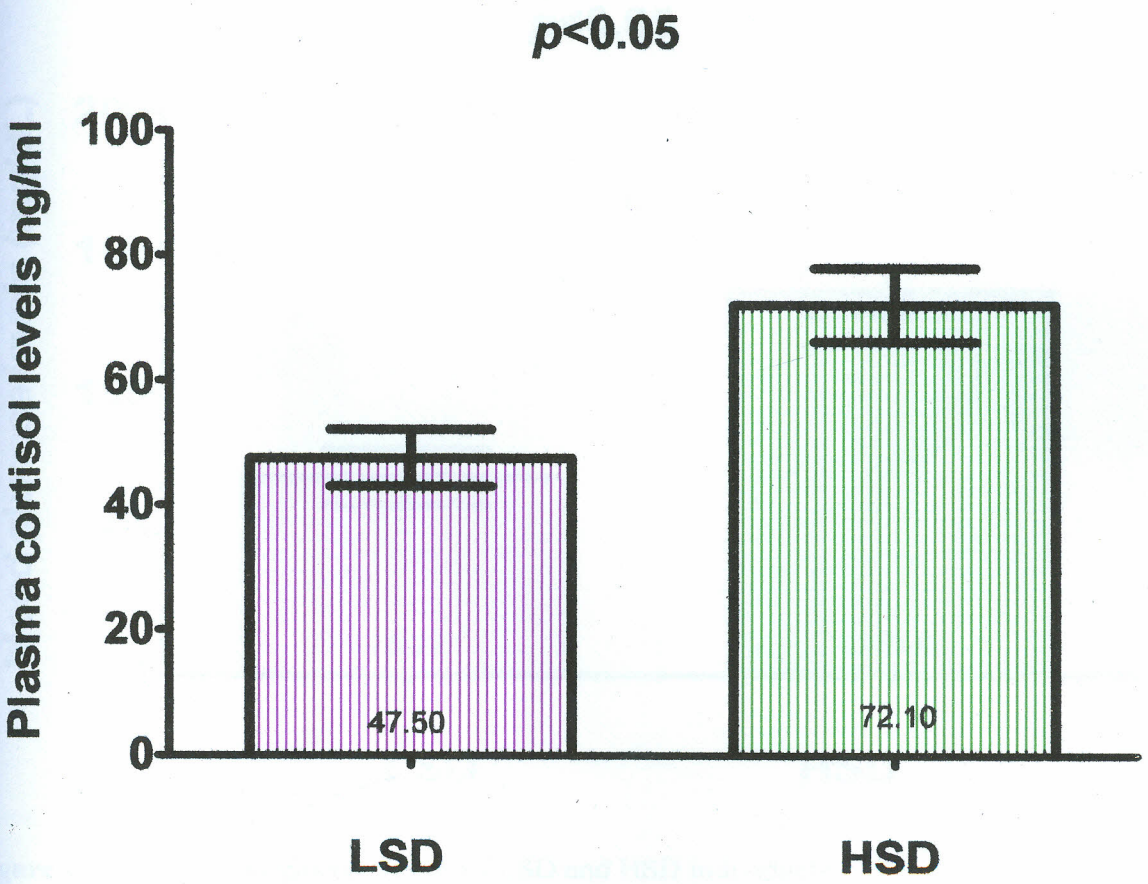

Figure 4.1. Mean plasma cortisol levels of fish subjected to low stocking density (LSD) and high stocking density (HSD) treatments.

4.2.2 Effect of high stocking density on blood glucose levels and haemoglobin concentration

4.2.2.1. Blood glucose levels

Blood glucose levels were raised in HSD individuals compared to LSD individuals (Fig. 4.2). The mean blood glucose levels of HSD group, $\bar{x}=136.00 \pm 2.17$ mg/dL, was significantly different from the mean blood glucose level of LSD group, 60.32 ± 2.55 mg/dL, at $p < 0.05$.

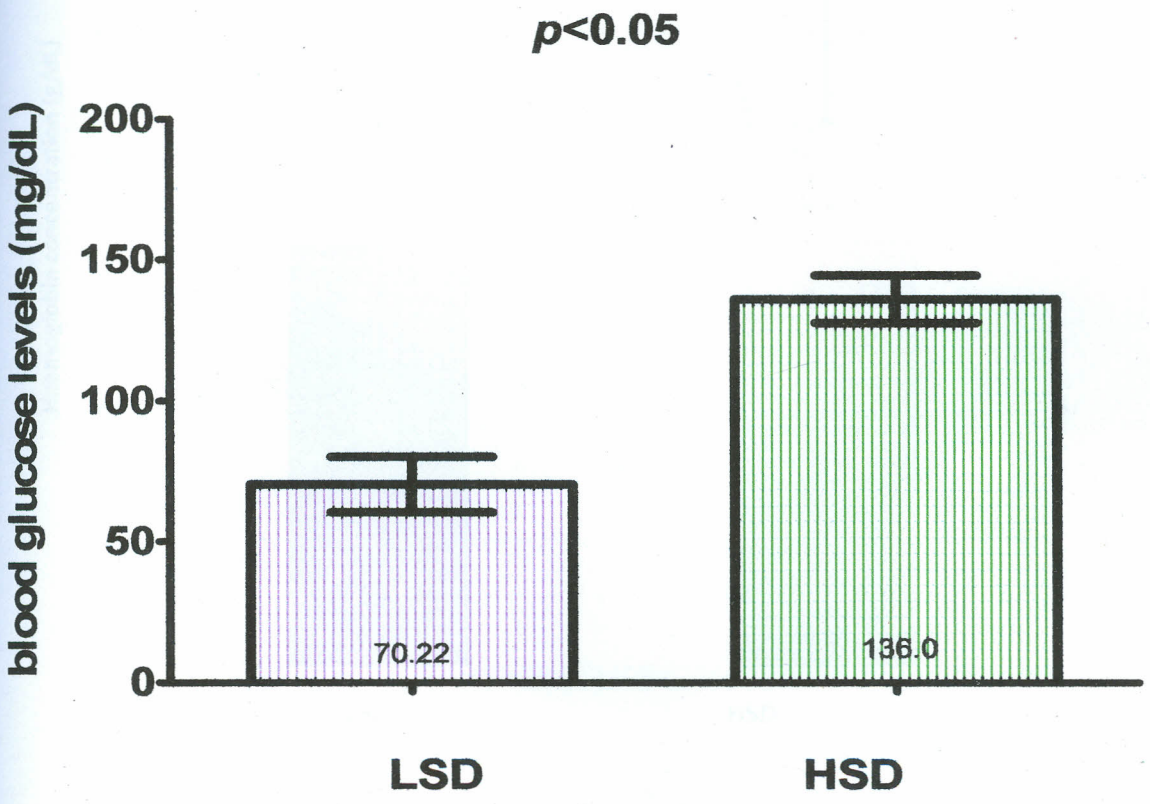

Figure 4.2. Mean blood glucose levels of LSD and HSD individuals

4.2.2.2. Haemoglobin concentration

High stocking density individuals had a higher mean haemoglobin concentration value than the LSD individuals. HSD mean haemoglobin concentration value of 9.86 ± 0.04 g/dL compared to 9.47 ± 0.10 g/dL as LSD mean for haemoglobin concentration (Fig. 4.3). Differences between the group means were significant ($p < 0.05$).

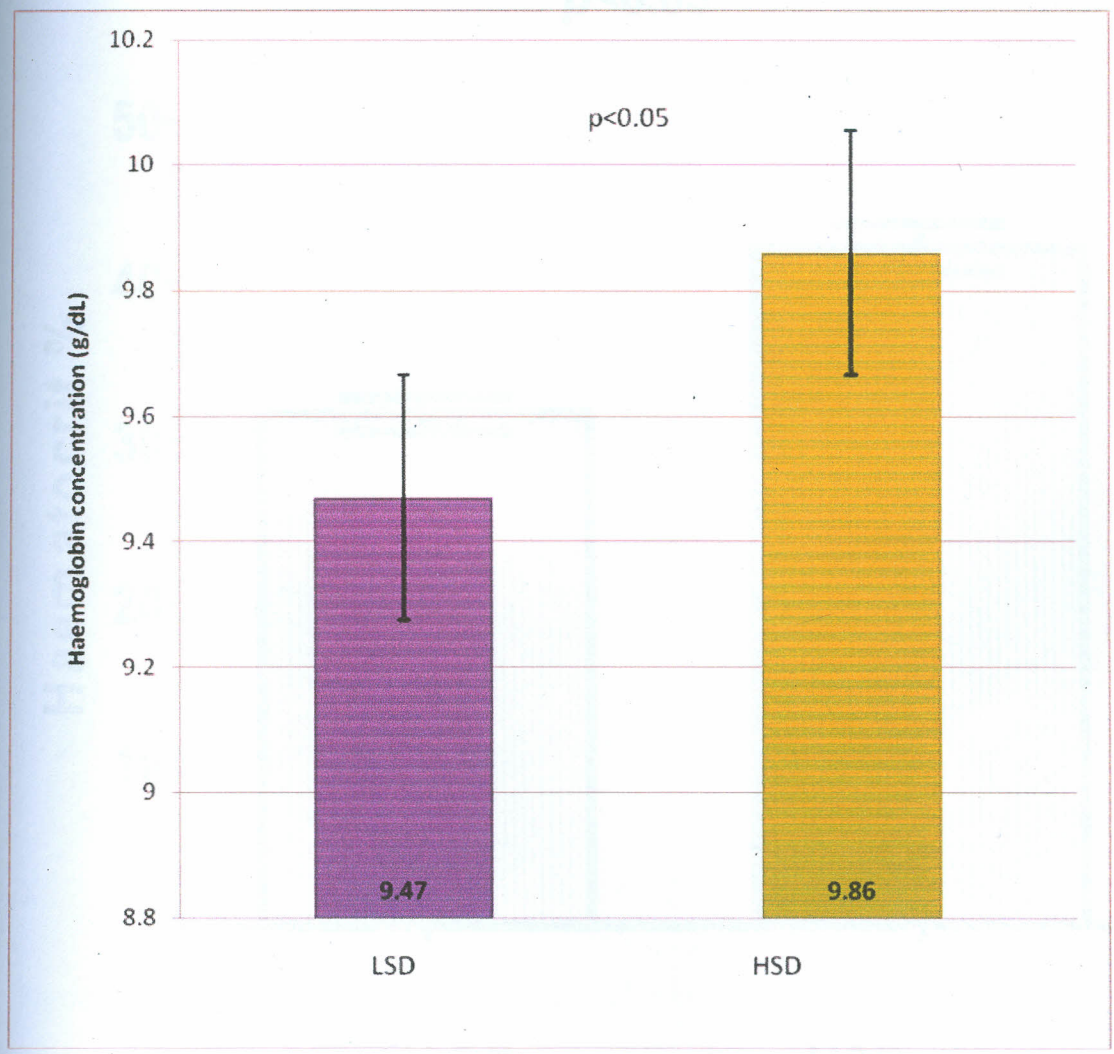

Figure 4.3. Mean haemoglobin concentrations of LSD and HSD groups

4.2.3 Effect of high stocking density on blood haematocrit

Haematocrit values were higher in HSD individuals compared to LSD individuals. HSD individuals had a mean haematocrit value of 42.08 ± 1.50 % whereas LSD individuals had mean value of 32.00 ± 1.00 %, $p < 0.05$ (Fig. 4.4).

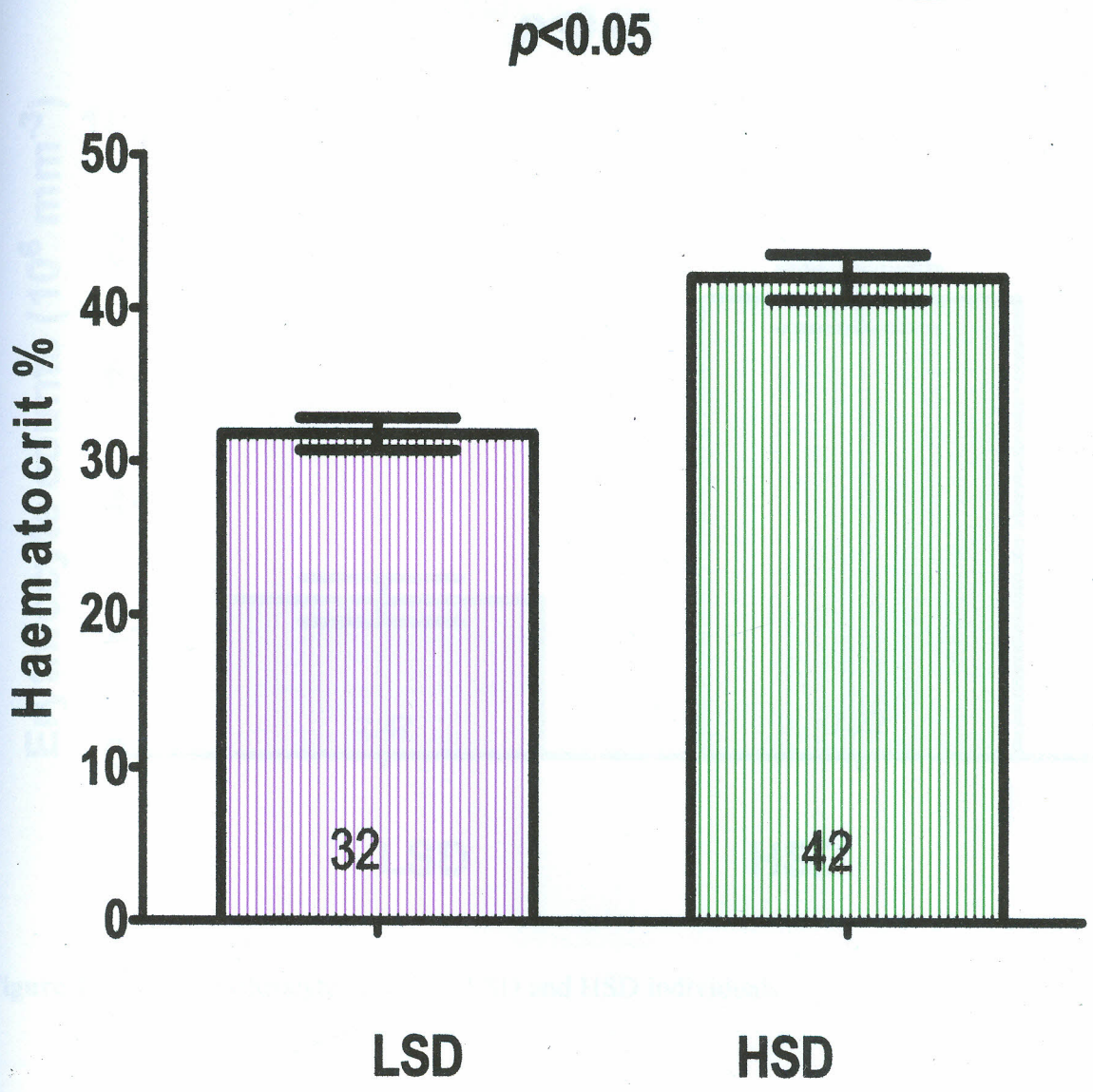

Figure 4.4. Mean haematocrit values of LSD and (HSD) individuals

4.2.4 The effect of high stocking density on erythrocytes count

There was a higher erythrocytes count in HSD individuals than in LSD individuals ($p < 0.05$). HSD individuals had a erythrocytes count of $7.27 \pm 0.48 \times 10^6$ mm^{-3} whereas LSD individuals had a erythrocytes count of $2.47 \pm 0.35 \times 10^6$ mm^{-3} (Fig. 4.5).

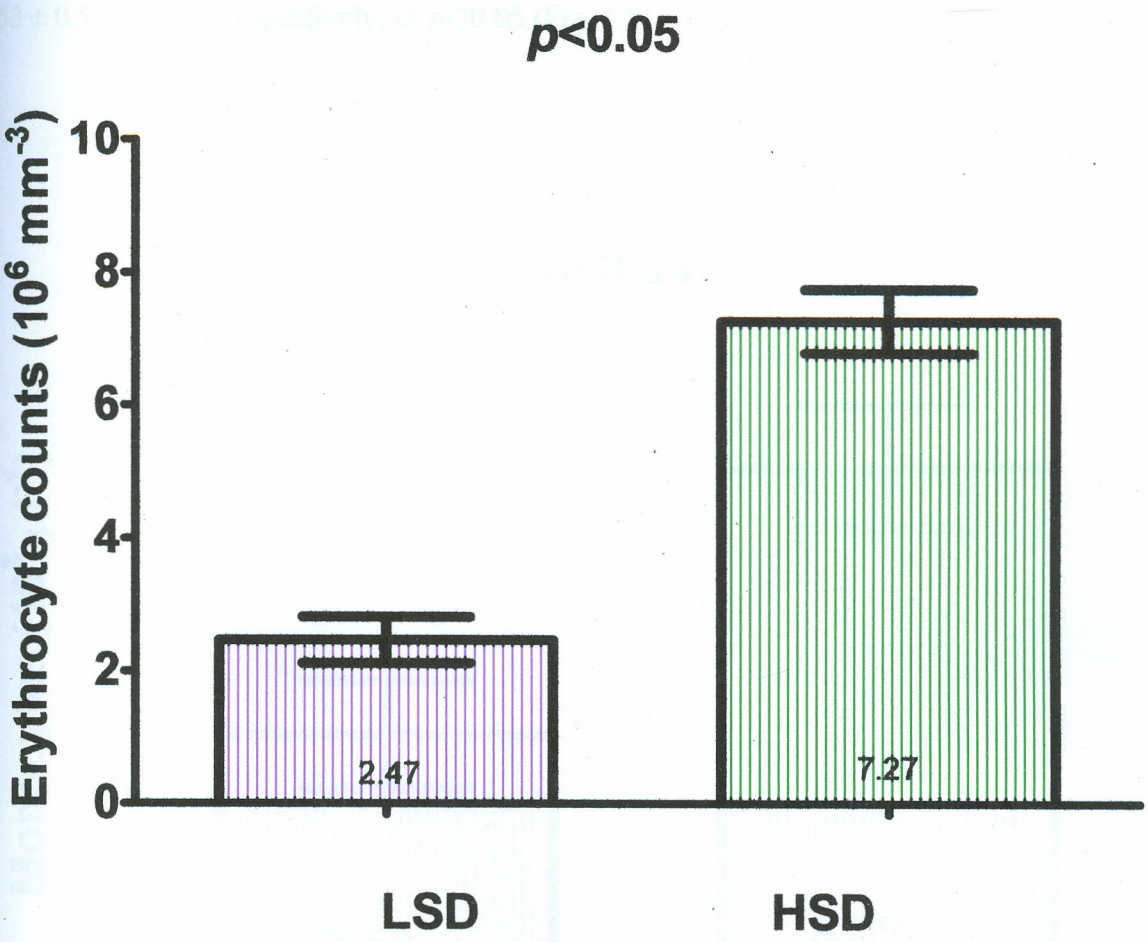

Figure 4.5. Mean erythrocytes count of LSD and HSD individuals

4.2.5 Effect of high stocking density on differential leukocyte (Monocytes, neutrophils and lymphocyte) count

4.2.5.1. Monocytes

High stocking density elevated the number of Monocytes in the fish blood ($p \square 0.05$). The HSD group had a significantly higher Monocytes numbers than LSD group, $5.07 \pm 0.58 \times 10^3\ \mu l^{-1}$ and $2.53 \pm 0.51 \times 10^3\ \mu l^{-1}$ respectively, at $p \square 0.05$ (Fig. 4.6).

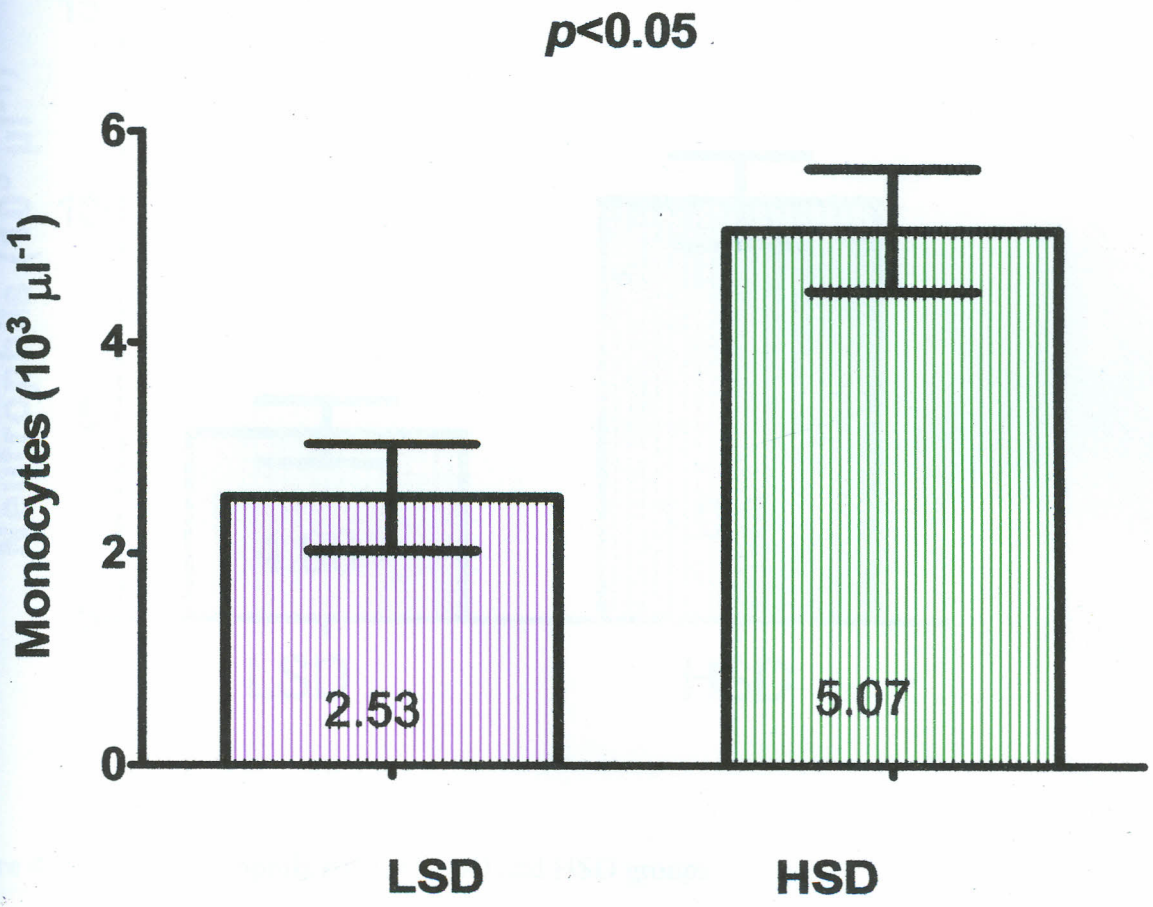

Figure 4.6. Mean monocytes numbers of LSD and HSD individuals

4.2.5.2. Neutrophils

The numbers of Neutrophils increased when the fish were subjected to HSD. Statistical analysis using student *t*-test at significance value of $p < 0.05$ revealed that there was a significant statistical difference in the mean Neutrophils values between HSD group, $10.33 \pm 1.12 \times 10^3\ \mu l^{-1}$, and LSD group, $4.60 \pm 0.77 \times 10^3\ \mu l^{-1}$, (Table 4.2, Fig. 4.7).

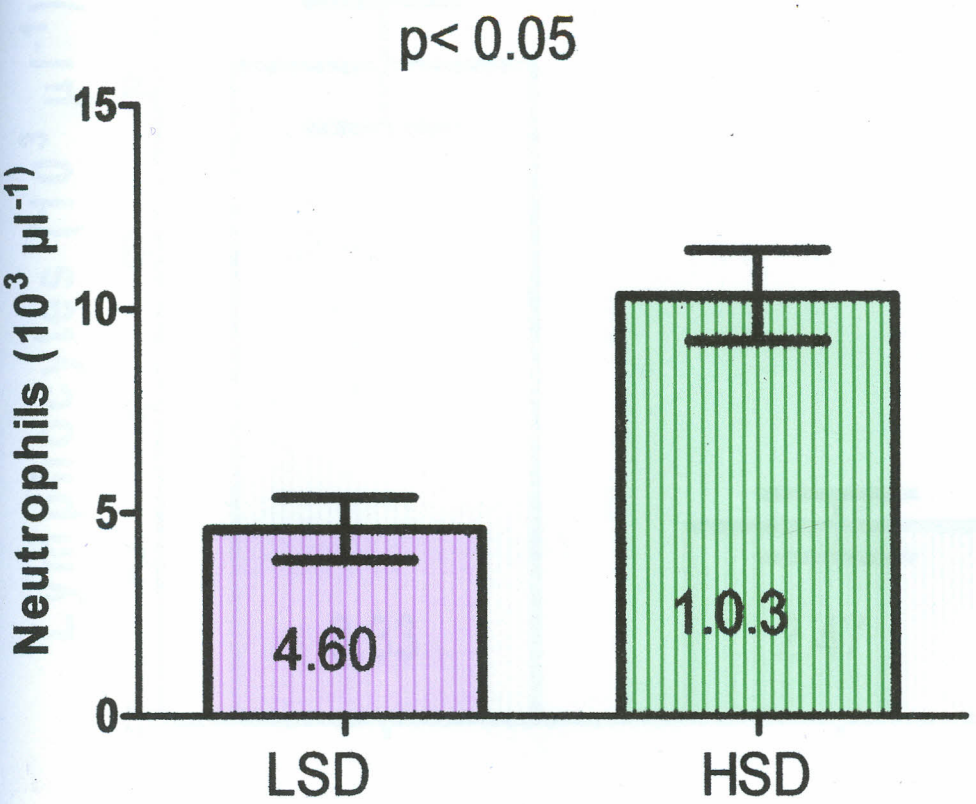

Figure 4.7. Mean Neutrophils count of LSD and HSD groups

4.2.5.3. Lymphocytes

The number of lymphocytes in HSD individuals was significantly lower than in LSD individuals at $p < 0.05$ (Fig. 4.8).

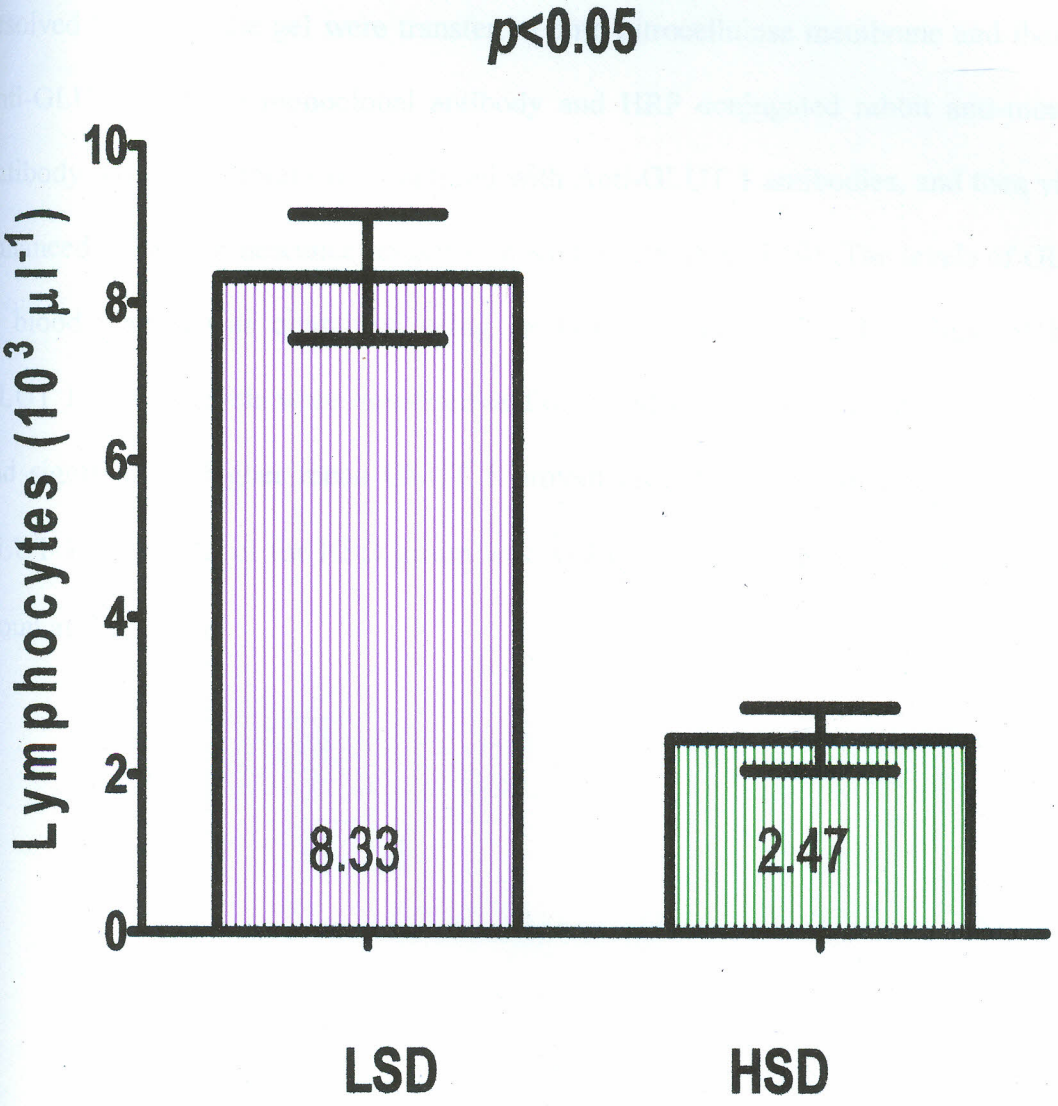

Figure 4.8. Mean lymphocytes count of LSD and HSD individuals

4.3 Resolution and quantification of GLUT 1 protein

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) technique was used to resolve blood plasma into fine bands (Fig. 4.9). SDS-PAGE gel revealed presence of bands with molecular weight of approximately 55 KDa, which is commensurate with molecular weight of GLUT 1 protein, in all samples both from HSD and LSD groups (Fig. 4.9, lanes 2 - 5). The resolved bands on the gel were transferred onto nitrocellulose membrane and then probed with anti-GLUT 1 mouse monoclonal antibody and HRP conjugated rabbit anti-mouse secondary antibody. GLUT 1 protein was captured with Anti-GLUT 1 antibodies, and then visualized with enhanced chemiluminescence reagents on Kodak film (Fig. 4.10). The levels of GLUT 1 protein in blood plasma was quantified using *ImageJ software* (National institute of health, USA). GLUT 1 protein levels were measured and recorded as relative band intensity. HSD individuals had significantly higher mean GLUT 1 protein level than LSD individuals ($p < 0.05$). Mean GLUT 1 protein level for HSD group was $\bar{x}=2.0 \pm 0.22$, compared to $\bar{x}=0.25 \pm 0.06$ of LSD group at (Fig.4.11)

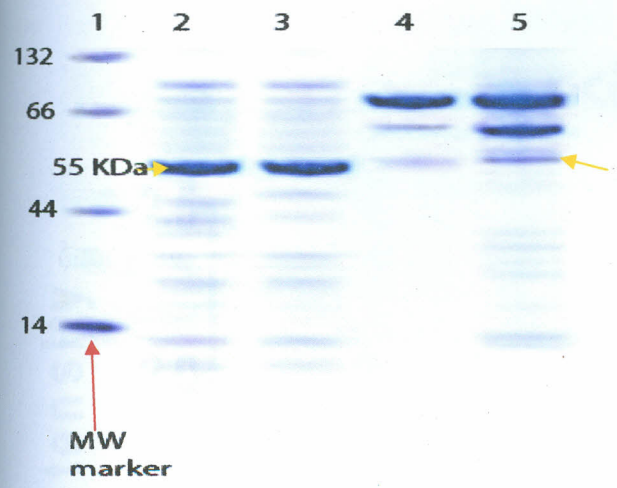

Figure 4.9. 12% SDS - polyacrylamide gel showing position of band(s) in lanes 2-5 with MW of approximately 55 KDa

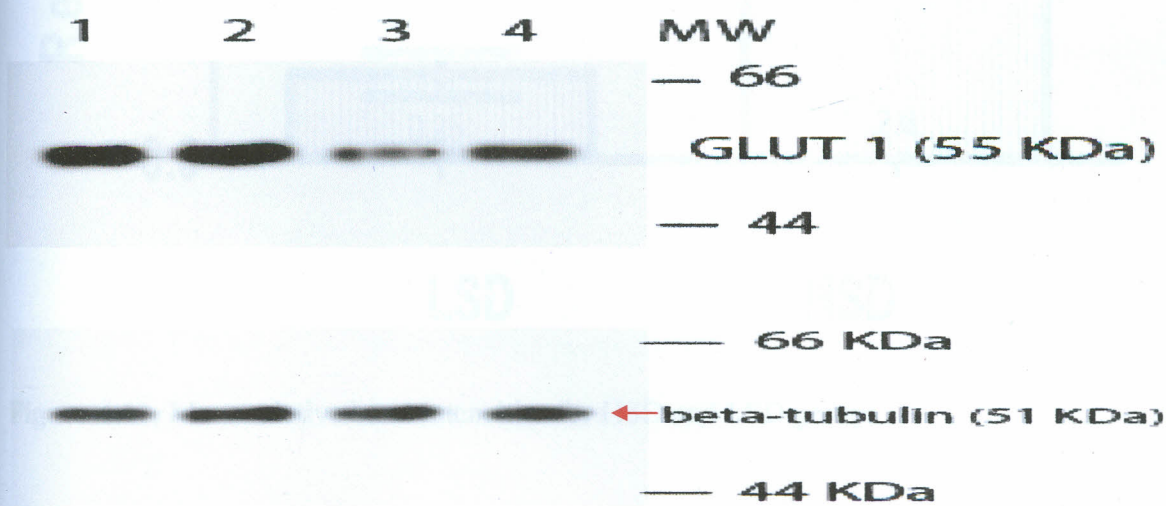

Figure 4.10: Western blot showing band intensities of plasma GLUT 1 protein (upper panel), which were compared to band intensities of β-tubulin (lower panel)

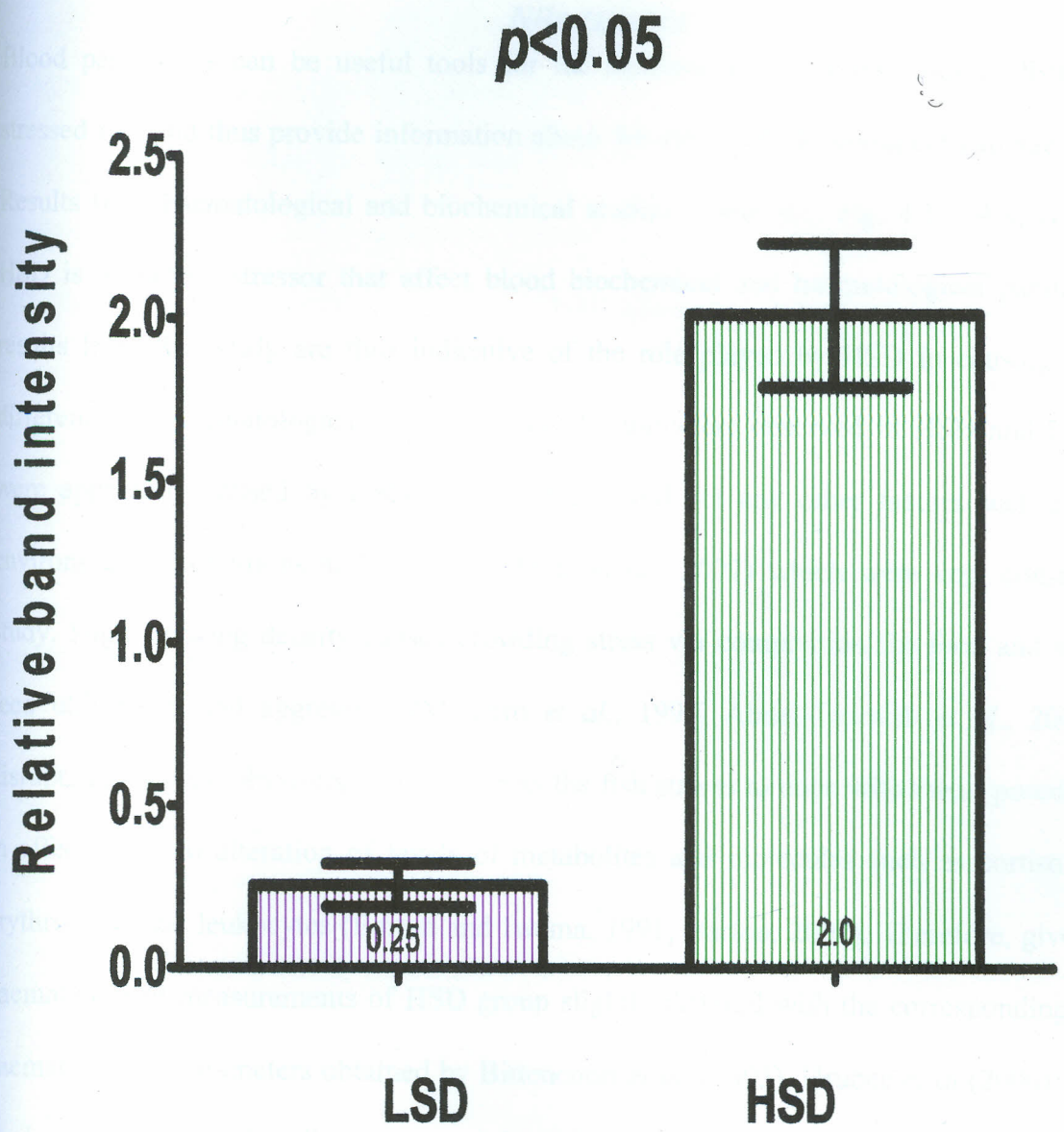

Figure 4.11. Mean relative band intensities for HSD and LSD groups

CHAPTER FIVE: DISCUSSION

5.1. Effect of HSD on haematological and biochemical characteristics of Nile tilapia

Blood parameters can be useful tools for the measurement of physiological disturbances in stressed fish and thus provide information about the extent of physiological damage in the host. Results from haematological and biochemical studies (Table 4.1; Fig. 4.1 – 4.8) confirms that HSD is a chronic stressor that affect blood biochemical and haematological parameters. The results from this study are thus indicative of the role played by HSD in causing stress. The differences in haematological and biochemical parameters observed in HSD and LSD groups were apparently caused by HSD-induced stress, and not any other factors such as age, sex, environmental conditions and feeding (Mauel *et al.*, 2007) which were kept constant in this study. High stocking density causes crowding stress via competition for food and space; poor feed utilization; and aggression (Montero *et al.*, 1999; Abdel-Tawwab *et al.*, 2005). Stress disrupts the normal physiology of the fish as the fish strives to cope with threat posed to it. This in effect leads to alteration of levels of metabolites and molecules such as cortisol, glucose, erythrocytes and leukocytes (Barton and Iwama, 1991; Barton, 2002). Therefore, given that the haematological measurements of HSD group slightly differed with the corresponding values of haematological parameters obtained by Bittencourt *et al.* (2003), Hrubec *et al* (2000) and Mauel *et al.* (2000) that are described as normal for Nile tilapia, it suffice to assert that the data obtained from HSD group are indicative of the presence of stress caused by HSD.

5.1.1. Effect of HSD on levels of plasma cortisol

High stocking density elevated mean cortisol level of the fishes. This finding is in agreement with Montero *et al.* (2001), who observed increased levels of plasma cortisol in gilthead seabream (*Sparus aurata*) held at high stocking density. HSD group had a mean cortisol value of 72.1±20.1 ng/ml, which was higher than the mean basal value (5 – 60 ng/ml) described by Auperin *et al.* (1997); and Barreto and Volpato (2001; 2006) normal for Nile tilapia (Fig 4.1). The high mean cortisol level in HSD individual indicates that the fish were stressed. The mean cortisol level for LSD group was within the range described by Barreto and volpato (2001; 2006) implying that LSD fish were not stressed. When fish is exposed to a stressor, interrenal cells are stimulated by ACTH to synthesize cortisol for circulation to various target cells (Montero *et al.*, 2001; Barton, 2002). The circulating cortisol will then increase oxygen demand in target cells and tissues in readiness for the alleviation of physical impairments caused by stress (Barton and Iwama, 1991; Mommsen *et al.*, 1999; Barton, 2002). Cortisol is also actively involved in the regulation of calcium absorption, blood pressure maintenance, anti-inflammatory function, gluconeogenesis, gastric acid and pepsin secretion, and immune function during stress in order to restore the normal homeostasis (Mommsen *et al.*, 1999).

(Schreck *et al.* 2001; Dobšikova *et al.*, 2009). In this regard secondary stress response can be considered an energy additive process, which enhances the level of glucose. The rise in blood glucose level produces a burst of energy which prepares the fish for an emergency situation. Glucose is an important fuel for metabolism and certain tissues for example brain, heart, blood cells, and gills rely primarily on glucose (Barton, 2002).

5.1.2.2. Effect of HSD on haemoglobin concentration

High stocking density individuals had a higher mean haemoglobin concentration value than the LSD individuals ($p < 0.05$). The mean haemoglobin concentration of both LSD and HSD groups were comparable to findings of Bittencourt *et al.* (2003) regarding Nile tilapia response to chronic stress. Therefore, both HSD and LSD group means fell within the normal reference value. This could be attributed to the fact Nile tilapia reacts to different stressors differently (El-Khaldi, 2000). For instance, the intensity of fish response to HSD stress is low compared to its response to heavy metals toxicity (Witeska, 2005). In chronic stress conditions, high haemoglobin concentration (such as the one observed in this study) is of necessity as it elevates blood pressure and oxygen transport efficiency that plays a role in preparing the fish to deal with the threat posed to it (Mommsen *et al.*, 1999; Abdel-Tawwab *et al.*, 2005).

5.1.3. Effect of high stocking density on blood haematocrit

Haematocrit values were higher in HSD individuals compared to LSD individuals, $p< 0.05$ (Fig. 4.4). The mean haematocrit in LSD group (31.56 ± 5.98 %) obtained in this study is within the range of the corresponding value described by Bittencourt *et al.* (2003) for *O. niloticus* (23.40 - 40.30 %), whereas the mean haematocrit value for HSD group (42.08 ± 1.50 %) surpassed the same specific ranges (Fig.4.4).

A high haematocrit mean value in HSD group is due to increased energy demand imposed by chronic stress. The high energy demand imposed on fish leads to alteration of physiological processes including respiration, blood pressure, erythropoesis, leading to a higher erythrocytes count that is coupled to oxygen transport efficiency, which is responsible for elevation of haematocrit (Abdel-Tawwab *et al.*, 2005). A higher haematocrit concentration in HSD individuals is responsible for increased oxygen carrying capacity and oxygen transport efficiency, which enhances oxygen supply various cells and tissues (brain and muscle).

5.1.4. The effect of high stocking density on erythrocytes count

There was a higher erythrocytes count in HSD individuals than in LSD individuals ($p < 0.05$). However, there was similarity of erythrocyte counts for HSD and LSD groups and erythrocyte count ($6.93 \pm 8.28 \cdot x\ 10^{6}\ mm^{-3}$) obtained by Bittencourt *et al.* (2003) for Nile tilapia. The rise in red blood cell numbers in fish held at HSD follows a pattern described in previous studies (Montero *et al.*, 2001; Bittencourt *et al.*, 2003; Dobsikova *et al.*, 2009) in which stress enhanced the numbers of erythrocytes. At the beginning of stress episode, pituitary, adrenal and thyroid glands regulate erythrocyte synthesis by releasing Adrenocorticotropic hormone, growth hormone, epinephrine, and nor-epinephrine which leads to increased erythropoietin concentration stimulating Erythrocytes production (Falcon *et al.*, 2007) hence increased number of erythrocytes in the blood of HSD group. Red blood cell is comprised of haemoglobin which binds oxygen molecules. Therefore, increase in number of erythrocytes is a strategy by the fish to transport oxygen to various cells and tissue. The oxygen molecules are used in the oxidation of substrates leading to the formation of energy, which is then used by the fish to cope with stress (Mommsen *et al.*, 1999).

5.1.5. Effect of high stocking density on differential leukocyte (monocytes, neutrophils and lymphocyte) count

5.1.5.1. Monocytes

High stocking density elevated the number of Monocytes in the fish blood ($p<0.05$). However, the two means were within the range of reference values for Monocytes obtained by Mauel *et al.*, (2007) implying that the similarity could due to difference in species of tilapia. This result is in agreement with findings of other studies in which an increase in monocytes were recorded in fish exposed to stress (Volpatti *et al.*, 1998; Sagstad *et al.*, 2007; Dobsikova *et al.*, 2009). It is thought that stress may induce migration of Monocytes to blood for circulation (Srivastava and Choudhary *et al.*, 2010). Monocytes have phagocytotic activities and are important in first line of host defence (Volpatti *et al.*, 1998).

5.1.5.2. Neutrophils

The numbers of Neutrophils increased when the fish were subjected to HSD ($p<0.05$). However, the two means were within the range of reference values of Neutrophils provided by Mauel *et al.* (2007). Implication of this result is that Nile tilapia (used in this study) and tilapia hybrid (used in previous study by Mauel) reacts differently to stress, and tilapia hybrid can cope well with stress. In this study, HSD stress could have induced the proliferation of neutrophils which corresponds to observation made by different researchers studying effect of stress on the levels of haematological indices (Volpatti *et al.*, 1998; Sagstad *et al.*, 2007; Dobsikova *et al.*, 2009). Increase in Neutrophils numbers of HSD individuals was probably cortisol-induced since this hormone prevents Neutrophils migration into the tissues and extends their life span by inhibition of apoptosis (Witseka, 2005; Srivastava and Choudhary, 2010). Neutrophils are phagocytotic in

nature thus plays an important role in the destruction of bacterial organism during and after stress (Schreck and Maule, 2001; Srivastava and Choudhary, 2010).

5.1.5.2. Lymphocytes

The number of lymphocytes in HSD individuals was significantly lower than in LSD individuals at $p < 0.05$ (Fig. 4.8). The data obtained from this study regarding the lymphocyte cells reveals that the number of lymphocytes decreased under chronic stress. This result is similar to the finding of Mauel *et al.*, (2007) obtained from tilapia. Decrease in number of lymphocytes during stress recorded in this study was also evident in other piscine species used in previous studies (Volpatti *et al.*, 1998; Sagstad *et al.*, 2007; Dobsikova *et al.*, 2009). During stress, lymphocytes are suppressed by hormones released from the adrenal gland (such as cortisol). Stress may also trigger blood lymphocytopenia (Srivastava and Choudhary, 2010; Mommsen *et al.*, 1999; Tavare-Dias *et al.*, 2007, and Rottmann *et al.*, 2007), thus reducing the number of lymphocytes. Therefore prolonged stress (and elevated cortisol levels) reduces the efficiency of the immune system [Fig 1.1], thereby increasing susceptibility to disease-causing organisms (Schreck and Maule, 2001).

5.2. Effect of high stocking density on expression levels of GLUT 1 protein

The results obtained in this study indicated that HSD individuals had significantly higher GLUT 1 protein plasma levels than LSD individuals ($p < 0.05$). HSD individuals had an eight-fold increase in GLUT 1 protein levels compared to LSD individuals (Fig. 4.11). Expression of GLUT 1 protein is regulated by extracellular stress and death-inducing agents (Samih *et al.*, 2000). In this study, HSD acted as a chronic stressor, and therefore modulated the levels of

stress-inducible GLUT 1 protein via the mechanisms explained here. First postulation is that HSD caused continuous stress that elevated levels of plasma cortisol. Cortisol produces many physiological changes in fish. Elevated level of cortisol imposes high energy demand on fish that leads to alteration of physiological processes including erythropoesis leading to a higher erythrocyte count (Abdel-Tawwab *et al.*, 2005). Falcon *et al.* (2007) observed that pituitary, adrenal and thyroid glands regulate erythrocyte synthesis by releasing Adrenocorticotropic hormone (ACTH), growth hormone (GH), epinephrine, and nor-epinephrine which lead to increased erythropoietin concentration stimulating erythrocytes production hence increased number of erythrocytes in the blood of HSD group, and this consequently explains the high levels of GLUT 1 protein, a transmembrane protein spanning the membranes of erythrocytes. Secondly, GLUT 1 levels of HSD group could have increased due to translocation of GLUT 1 from the intracellular pool to plasma membrane and by changes in the turnover rate of the transporters (Lund *et al.*, 1995). Translocation of GLUT 1 from the intercellular pool to plasma membrane is by enzyme phosphatidylinositol 3 kinase (PI–3 kinase) catalysis which regulate various steps in receptor-dependent endocytic trafficking (Samih *et al.*, 2000). PI-3 kinase that participates in the regulation of cell growth and metabolism is activated by several tyrosine kinase receptors including the insulin receptor (IR). PI-3 kinase interacts with IR protein leading to recruitment of GLUT 1 protein from intracellular pool (Medina *et al.*, 2000).

There was high blood glucose level corresponding to high GLUT 1 protein level in blood. This could imply that GLUT 1 protein is not efficient in transportation of glucose molecules in fish blood. There are some few reasons advanced as to why the role of GLUT 1 in glucose homeostasis is much less significant in teleost fishes. Unlike in mammals where glucose clearance rate is rapid, teleost fishes appear not to have a mechanism to move glucose from bloodstream into muscle and fat tissue (Wright *et al.*, 2000). Teleost fishes are thought to be glucose intolerant species due to limitation of GLUTs and resistance to glucose lowering effect of insulin in the peripheral tissues. The notion that GLUT 1 role in glucose metabolism in teleost fish is less significant is simply untrue as it has been shown that the glucose clearance rate is time-dependent and improves gradually (Wright *et al.*, 1998). GLUT 1 being the major transporter protein in erythrocyte is responsible for glucose transport in fish blood and thus GLUT 1 takes comparatively longer time to move glucose molecules from blood to peripheral tissues (Diaz *et al.*, 2009).

CONCLUSION AND RECOMMENDATION

6.1 Conclusions

- High stocking density modulated the levels of GLUT 1 protein in blood of Nile tilapia. GLUT 1 protein is a stress-inducible protein whose blood level is regulated by stress. Therefore, plasma level of GLUT 1 protein can be used as a stress biomarker.

6.2 Recommendations

1. Recommendations for future studies: RT - qPCR based study should be done to analyze the effect of HSD induced stress on the levels of GLUT 1 in various tissues (brain, kidney, liver, and heart) expressing GLUT 1. The threshold values for the expression of GLUT 1 mRNA at which stress begin to occur should be determined and adopted as reference values.

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