HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS AS ALTERNATIVE MARKERS OF STOCKING DENSITY- INDUCED STRESS IN CULTURED NILE TILAPIA, (*Oreochromis niloticus* Linnaeus, 1758) UNDER CULTURE CONDITIONS.

BY

ELIJA ODHIAMBO ONYANGO

ATHESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CELL AND MOLECULAR BIOLOGY

SCHOOL OF PHYSICAL AND BIOLOGICAL SCIENCES

MASENO UNIVERSITY

DECLARATION

I declare that this is my original work and has not been submitted in part or whole for an award in any institution to the best of my knowledge. All referenced sources are duly quoted, and any semblance observed is coincidental. This thesis or any part of it may not be reproduced without written permission from the author, my supervisors or Maseno University presented for the award of degree in any other University.

Signature……..Date……..

Onyango Elija Odhiambo
(PG/MSC/00008/2008)

This thesis has been submitted for examination with our approval as university supervisor and the academic advisor respectively.

Signature……..Date……..

Prof. David Miruka Onyango, PhD
Department of Zoology,
School of Physical and Biological Sciences,
Maseno University,
Maseno, Kenya.

Signature……..Date……..

Dr. Paul Oyieng' Ang'ienda, PhD
Department of Zoology,
School of Physical and Biological Sciences,
Maseno University,
Maseno, Kenya.

ACKNOWLEDGEMENT

I am sincerely grateful to my mentors, the late Prof. Eliud Nyandago Waindi, Prof. David Miruka Onyango, Dr. Paul Oyieng' Ang'ienda and Dr. Patrick Okoth for their mentorship during the entire period of undertaking this study. The Department of Zoology at Maseno University and the Kenya Medical Research Institute (KEMRI) are acknowledged for providing laboratory space, equipment and technical expertise that enabled the success of this study. Dr. Cyrus Ayieko of the Department of Zoology - Maseno University is highly appreciated for providing valuable scientific and technical assistance during sample analysis. The former Chairman, Department of Chemistry – Maseno University, the late Prof. Job Isaac Jondiko for allowing me to use the chemistry laboratory space and equipment's for the analysis of samples. I wish to also thank Prof. David Miruka Onyango. Dr. Paul Oyieng` Angienda and Dr. Patrick Okoth for the scientific and technical assistance in the development and publication of this Thesis, a Research Article "Stocking Density Induced Stress on Plasma Cortisol and whole Blood Glucose Concentration in Nile Tilapia Fish (*Oreochromis niloticus)* of Lake Victoria, Kenya" (Odhiambo *et al*., 2020) published in the International Journal of Zoology. Finally, I would like to give thanks to everyone who contributed to this study but whose individual mention was an inadvertent omission. Most importantly, I am grateful to God for His insurmountable support.

DEDICATION

I dedicate this thesis to my wife, Caroline Wambui Kariuki, My sons James and Peter, my motherBendeta.

ABSTRACT

Fish and fisheries play a significant role in meeting nutritional food security, more so of the poor communities in the low and middle income countries (LMIC).*Oreochromis niloticus*isone the most valuable warm water fishes used in aquaculture systems. Various stressors trigger specific stress responses in *O. niloticus*. High stocking density (HSD) causes persistent stress response, which brings about inhibited fish breeding, growth, and decreased immune capacity thus decreased yield. Studies have been done in other regions of the world to evaluate the various hematological and biochemical parameters in *O. niloticus* as markers of stress, thus this study evaluated four specific parameters and to customize them for use by *O. niloticus* farmers in the Counties boardering Lake Victoria in Kenya. Two specific objectives were evaluated in this study namely; to determine the relative hematological parameter; erythrocyte count in cultured *O. niloticus*, subjected to stocking density induced stress and to determine the relative biochemical parameters; plasma cortisol, whole blood glucose, and blood electrolytes concentrations in cultured *O. niloticus*, subjected to stocking density induced stress. Stocking rates for the aquaria was done by matching all the fish for body weight/standard mass, Mean± SD (15 ± 1 g) thus, 150 g and 300 g of body weight/ 0.05 m^3aquarium for low stocking density (LSD) and HSD respectively both in triplicates. The fish were cultured for 21 days following 3 days of acclimatization to the laboratory conditions after being obtained from the University`s fish rearing facility by seining technique. The fish were fed on a carbohydrate based feed of chick mash at 18 % protein supplemented with crushed *Rastrineobola argentea* to 25 % protein at a feed portion of 10 g/kg of life body weight. Blood samples were drawn using cardiac puncture technique from fish anaestethised with 2 – phenoxyethanol at 0.30 $ml.l^{-1}$ from each aquarium (n = 5) using a large sieve. Erythrocyte counts (n = 18) were assayed for using hemocytometer. Plasma cortisol concentration (ng/ml) (n =18) levels were assayed for by use of **E**nzyme-**L**inked **I**mmuno**s**orbent **A**ssay. Whole blood glucose concentration levels (n = 30) were determined using a hand-held One Touch Ultra glucose meter (MD-300) and test strips. Electrolyte concentrations (n =18) were assayed using Sherwood Flame Photometer 410. Whole blood glucose analysis revealed statistical ($P< 0.05$) difference in the means in HSD and LSD *O. niloticus* groups. Mean plasma glucose concentration was statistically significantly ($P \leq 0.01$) higher for HSD than LSD *O. niloticus* groups at mean ± SD, 96.84 ± 5.28mgd^{-1} and 76.80 ± 5.92 mgd^{-1}respectively. One-way Analysis of Variance (ANOVA) was done on the data collected and comparison of significant differences in means done between LSD and HSD at 0.01%. Plasma cortisol levels revealed statistically ($P \leq 0.01$) significant values of HSD at mean ± SD, 6.35 ± 0.89ng/ml and 4.49 ± 1.08 ng/ml respectively. One way ANOVA analysis of the true electrolyte means for the LSD and HSD*O. niloticus* groups revealed significantly ($P < 0.05$) higher mean at mean ± SD (1.63 ± 0.18 mmol/l) $_{HSD}$ and (1.11 ± 0.08 mmol/l)$_{LSD}$ for Na^+, (0.73 ± 0.03 mmol/l)$_{HSD}$ and (0.42 ± 0.02 mmol/l)$_{LSD}$ for K^+ with no statistical ($P< 0.05$) difference at mean ± SD (0.13 ± 0.00)$_{HSD}$ and (0.13 ± 0.00)$_{LSD}$ for Ca^{++}.One-Way ANOVA analysis revealed significant ($P < 0.05$) difference in the erythrocyte count means in LSD and HSD *O. niloticus* groups at mean ± SD, 7.01 ± 0.77 x 10^6 mm^{-3} and 3.36 ± 0.63 x 10^6 mm^{-3} respectively. Overally, the findings of this study demonstrate that high HSD increase erythrocyte count, plasma cortisol, whole blood glucose, and Na^+ and K^+ concentration in *O. niloticus* fish indicating a marked increase in stress levels. Elevated erythrocyte count, plasma cortisol, whole blood glucose, and Na^+ and K^+ concentrations can be used as alternative biomarkers for acute stress in *O. niloticus* produced under aquaculture systems. The findings of this study can help improve aquaculture practices on management of chronic stress in *O. niloticus* and related Cichlids under industrial aquaculture production through timely diagnosis and deployment of appropriate mitigation measures.

TABLE OF CONTENTS

ABBREVIATIONS AND ACRONYMS

12L: 12D	12Light: 12Darkness
ACTH	Adrenocorticotropic Hormone
ADCP	Aquaculture Development and Coordination Programme
ANOVA	Analysis of Variance
APS	Ammonium Persulfate
B/B_o	Maximum Binding
BPI	Brain-Pituitary - Interrenal Axis
BSCC	Brain-Sympathetic - Chromaffin Cell Axis
CAs	Catecholamines
CP	Crude Protein
CRF	Corticotrophin Hormone
CRH	Corticotrophin-Releasing Hormone
DWG	Daily Weight Gain
Epi	Epinephrine
GH	Growth Hormone
Glut 1	Glucose Transporter 1
HPA	Hypothalamus-Pituitary-Adrenal Axis
HPI	Hypothalamic-Pituitary-Interrenal Axis
HSD	High Stocking Density (0.006 kg body mass/litre)
Hsp 70	Heat Shock Protein 70
HSPs	Heat Shock Proteins
IgM	Immunoglobulin M
LMIC	Low and Middle – Income Countries
LSD	Low Stocking Density (0.003 kg body mass/litre)
MT	Metric tones
MS-222	Tricane Methane Sulfonate
NE	Norepinephrine
PAGE	Polyacrylamide gel electrophoresis
PVN	Para-Ventricular Nucleus
SDS	Sodium Dodecyl Sulphate
TK	Tyrosine Kinase
WAT	White Adipose Tissue.

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1. Background Information

Fish and fisheries play a significant role in meeting nutritional food security, more so of the poor communities in LMIC (Ridha 2006). More than 2 billion people worldwide obtain at least 20% of their animal protein consumption from fish (Hassanien and Gilbey, 2005; Acosta and Gupta, 2009). Nile tilapia (*Oreochromis niloticus*) is one of the most valuable warm water fishes used in aquaculture systems (Charo – Karisa *et al.,* 2005) and only the second most popular farmed fishes after Carps (Acosta and Gupta, 2009; El-Sayed, 2002; Getinet, 2008). They are recognized as one of the most important species in tropical and sub-tropical aquaculture (Hassanien and Gilbey, 2005; El-Sayed, 2002; Li *et al.,* 2001) which provides food, employment, and contributes to a country`s economy (Luomba *et al*., 2013). Nile tilapia have a mild white flesh that appeals to customers, making them economically important fish (Masterson, 2007) and the most desired by Lake Victoria Community (Njiru *et al.,* 2006) amongst other consumers of fish. Volpato *et al*., (2003) notes that, the fish important model for studies on social stress induced by overstocking.

Stocking density is treated as an important factor in gauging the productivity of fish aquaculture systems (Rebl *et al*., 2017) and the most commonly abused fish rearing practice with wrong perception that it leads to higher yields thus its choice for this study. Crowding is considered amongst the most impactful stressors influencing the physiology of fish and by extension, the status of aquaculture`s well - being(Barton, 2002) and it is a common husbandry practice in aquaculture (Abdel-Tawwab *et al*., 2005;Aketch*et al*., 2014) with the culture systems categorized as; a) extensive i.e., earth ponds, lagoons, small pens b) semi-intensive i.e., earthen, liner, concrete ponds and cages/pens, polyculture and/or monoculture and c) intensive i.e.,

tanks(flow/recirculated), cages and bio flock array systems (Mungutiet *al.*,2021). High stocking density impacts negatively on growth and feed utilization by fish (Ridha 2006; Abou *et al*., 2007). High stocking densities has been associated with reduction on feeding activity and growth rates in *Oncorhynchus kisutch*, and *Oncorhynchus mykiss* but have a positive impact on both parameters in *Salvelinus alpinus* (Wendelaar-Bonga, 1997). It increases the levels of metabolites notably, urine and faeces in cultured *Chrysichthys nigrodigitatus* (Pangni *et al*., 2008). This stress response changes water quality (Abou *et al.,* 2007) thus further subjecting fish to chemical stressors (Pangni *et al*., 2008; Francis-Floyd, 2009). Various studies have demonstrated an inverse relationship between stocking density and growth rate (El-Sayed, 2002; Ridha, 2006) with the growth rate taken as a measure of the percent increase in fish weight per unit time (Crane *et al.,* 2019), mainly due to social interactions (El-Sayed, 2002). Densities of 1.0 and 2.0 kgm^{-3} induce stress in *O. niloticus* fingerling under laboratory conditions (Barcellos *et al.,* 2001). In *O. niloticus*, crowding stress negatively affect survival, percentage weight gains and specific growth rate in fry (El-Sayed, 2002) and growth performance in adults (Ridha, 2006)**.** High stocking density subjects fish to chronic stress (Pickering, 1993; Barcellos *et al*., 2001; El-Sayed, 2002;Goncalves-de-Freitas and Mariguela, 2006) which causes impaired fish growth (Pickering, 1993; El-Sayed, 2002), impaired growth mostly associated with the aggregation of food available (dietary energy) for consumption by fish through the physiological adjustments triggered by stress (El-Sayed, 2002).Stocking density and social interactions between conspecific fish is reported to have significant influence on stress with hierarchical organization (Barcellos *et al*., 2001; Cnaani et al., 2004). Stress is considered to be a generated response but it can be modulated by specific stressor conditions. One of the most frequent causes of chronic stress is social interaction among members of the same species. Stressors are intrinsic or extrinsic stimuli that threaten or disturb the dynamic equilibrium

of animal organization called homeostasis (Wendelaar-Bonga, 1997). The mechanism by which stressors elicit stress response in fish work by the activation of the hypothalamus-pituitary-adrenal (HPA) axis (Moreira & Volpato, 2004) for integration of the adaptive responses to stress thus initiating a number of behavioral and physiological changes that improve the fish's chances of survival when faced with homeostatic challenges(Smith and Vale, 2006). The heterogeneity of stress responses varies within individual's fishes, and stressors become multimodal in terms of typology, source and effects, as well as the responses that each individual elicits to cope with the disturbance (Balasch and Tort, 2019).

Chronic stress is reported to cause hyper-metabolism characterized by enhanced glycolysis, gluconeogenesis, altered glucose uptake and reduced glycogenesis thus predisposing fish to the development of chronic illnesses accompanied by metabolic dysregulation (Nirupama *et al*., 2018). Stressors cause distinct stress responses in *O. niloticus* (Barreto and Volpato 2006) such as harmful effects on a number of physiologic functions comprising of growth, reproduction, metabolism, immune competence, behavior, personality development (Tsigos *et al*., 2016)and elevated plasma cortisol concentration levels, cortisol being the main hormone that activates glucose (Wendelaar Bonga 1997; Gomes*et al*., 2003;Abreu *et al*., 2009)therefore elevating glucose levels (Volpato and Fernandes 1994; Barcellos *et al*., 1999; Abreu et al., 2009).Fish respond to stress by increasing circulating cortisol (Wendelaar Bonga 1997; Abreu *et al*., 2009) which mediates stress-associated response (Kalamarz-Kubiak, 2017) and glucose (Abreu *et al*., 2009). Cortisol is released by interrenal cells of the head kidney during activation of the hypothalamic-pituitary-interrenal (HPI) axis in response to chronic stressors (Kalamarz-Kubiak, 2017) such as long persistent stress (Nirupama *et al*., 2018) which leads to decreases in effects such as growth rate, disease resistance and reproductive success (Iwama *et al.*, 2004). Elevated plasma cortisol

(Barcellos *et al*., 1999; Martinez-Porchas *et al*., 2009) and glucose levels (Wendelaar Bonga 1997; Cnaani *et al*., 2004; Barreto and Volpato 2006;Martinez-Porchas *et al*., 2009) are used as indicators of stress when stimuli stressors are noticed to illicit an integrated response to stress in fish through spontaneous or extreme variations in i) the physical environmental factors such as; contaminants, temperature, turbidity and salinity ii) animal interaction factors such as; spawning, predation, parasites, intensive competition for space, food, or sexual partners iii) human interference comprising of aquaculture practices such as; confinement, netting, handling, transport, repeated capture and crowding iv) water pollution as a result of; water levels, low water pH, heavy metals, and organic chemicals (Wendelaar Bonga 1997;Dykstra and Kane, 2000; Conte, 2004) if diminishing innate response to frequency of repeated exposure to the aforementioned variations doesn't occur. This study investigated the haematological (i.e., erythrocyte count) and biochemical (i.e., plasma cortisol, whole blood glucose and electrolytes) parameters as alternative markers for stocking density (body weight at 300 g per 0.05 m^3 of water) induced stress in *O niloticus* reared under aquaculture systems.

1.2. Statement of the Problem

Fish farming involves adapting standard practices and procedures revolving around addressing; handling, water levels, confinement, and crowding. These procedures could act as stressors when habituation does not occur. High stocking density coupled with other unstandardized fish rearing parameters such as; feed, water levels, temperature, oxygen concentration etc., subjects fish to chronic stress with correct stocking density being critical in ensuring proper fish welfare. Many fish farmers have a misguided understanding that overstocking of fish leads to more earnings contrary to the scientific fact that, overstocking creates stress which leads to increase in mortality thus compromised yields. Stressors cause distinct stress responses in *O. niloticus* with stocking

density inversely relating to feeding activity and growth rate. This pauses the problem of reduced yields per cubic meter in ponds and cages as a result of reduced body size, longer duration taken to maturity and the escalated cost of production in regards to feeds and maintenance arising from HSD. Studies on the impact of high stocking density induced stress on the expression of hematological and biochemical parameters as biomarkers of stress in different species of pieces including Nile tilapia thus become critical and forms the basis on which these investigations were premised. This study, therefore sought to investigate selected hematological together with biochemical parameters as biomarkers of stress that can help inform policy on the management of stress in *O. niloticus* and other related Cichlids under industrial aquaculture systems in Lake Victoria basin.

1.3. Study Objectives

1.3.1. General Objectives

To evaluate hematological; erythrocyte count and biochemical parameters; plasma cortisol, whole blood glucose, blood electrolytes as alternative biomarkers of stocking density induced stress in cultured Nile tilapia, (*Oreochromis niloticus* Linnaeus, 1758).

1.3.2. Specific Objectives

1. To determine the relative erythrocyte count in cultured *Oreochromis niloticus*, subjected to stocking density induced stress.

2. To determine the relative concentrations of plasma cortisol, whole blood glucose and blood electrolytes in cultured *Oreochromis niloticus*, subjected to stocking density induced stress.

1.3.3.Null Hypotheses

1. There is no significant difference in the relative erythrocyte count in cultured *Oreochromis niloticus*, subjected to stocking density induced stress.
2. There is no significant difference in the relative concentrations of plasma cortisol, whole blood glucose and blood electrolytes in cultured *Oreochromis niloticus*, subjected to stocking density induced stress.

1.4. Justification of the Study

Nile tilapia production under culture **sys**tems i.e., commercial pond and cage culture systems are gaining traction in the Lake Victoria basin, Kenya, with a total population of 4.855946 million (KNBS, 2019) and has largely boosted production of farmed Tilapia. A survey conducted in Kenya, in 2017 recorded a rapid rise in cages from 1,663 (in 2015 – 2016) to 3,696 with production estimated at 3.18 metric tons (MT) with a market value of 9.6 million USD (KEMFRI 2018).However, KNBS (2019) records a significantly depressed performance in pond-based aquaculture production in Kenya, with total fish output dropping from 24,096 MT in 2014, 18,656 MT in 2015, 14,952 MT in 2016, 12,356 MT in 2017, 15,320 MT in 2018 and to 18,542 MT in 2019attributed to poor fish production practices. Furthermore, an investigation in the beaches of the five riparian counties of Lake Victoria, Kenya, up to 75 % of cage investors had no standard tilapia stocking density, with 10 % of them using increase in yields to estimate cage stocking densities while 80 % of the cage investors were found to be using different feeding regimes. The aforementioned study`s most successful cage investors realized maximum resultant production yields of 10 – 14 kg body mass/m^3 within 6-8 months (Ombwa *et al*., 2018) which was significantly lower than up to 330 kg body mass/m^3recorded from an intensive tilapia cage culture systems in four months (Rojas and Wadsworth, 2007).The varied stocking densities and feeding regimes

predisposed the caged fish to negative effects of stress. Nile tilapia is susceptible to stress caused by poor production management practices such as handling, low water levels, confinement, crowding amongst other practices. Stress is amongst the factors that compromise both fish growth performance and immune condition; hampering health and welfare (Portz *et al*., 2006) negatively affecting Tilapia through reduced productivity in the number of fingerlings, reduced growth rate, the feeding habits resulting into body size that does not attract economic value in aquaculture systems. Stress detection and its management policies are still very poor both in Lake Vicoria basin and by extension in Kenya, given the fact that Nile tilapia production under culture systems is yet to be properly understood in aquaculture systems. High stocking density on which this study is premised has negatively affected the control of fish size and productions which are important in meeting market demands in aquaculture by determining production and profitability since they directly affect Tilapia survival, growth, behavior, health, water quality, and feeding (Abdel-Tawwab *et al*., 2005; Oke and Goosen, 2019).Therefore, the management of high stocking density and other stressors `induced stress in *O. niloticus* reared under cultured systems becomes critical so as to avoid compromising yields for maximum profitability.

1.5. Significance of the Study

Firstly, the findings of this study can help inform policy on the management and development of strategies that will maximally reduce stress in *O. niloticus* and other related Cichlids under industrial aquaculture systems. Secondly, the findings of this study will buttress both the existing management policies and the scientific body of knowledge already generated on understanding stress and its effect on the expression of erythrocyte, plasma cortisol, whole blood glucose and blood electrolytes in tilapia subjected to stocking density and other stressors` induced stress under culture systems.

1.6. Limitations of the study

The present study had the following potential limitations;

1. Complete elimination of handling induced stress during sampling may not have been achieved despite a one minute blood sample extraction period with potential effect on plasma cortisol and blood glucose concentration levels.
2. Precision error could have occurred in the Erythrocyte count since the study assumed even distribution of the red blood cells on the outer lines of the improved Neubeur chamber used, which had the potential of causing a large shift in the counting result.
3. The scope of the haematological; erythrocyte count and biochemical; plasma cortisol, whole blood glucose, and electrolytes parameters studied as biomarkers of stocking density induced stress in *O. niloticus* studied was limited since the study did not consider a number of other haematological, biochemical, and cellular Heat shock protein markers of stress in fish.
4. The sample size was limited due to death of fish apparently from stress associated with high stocking density in the overcrowded aquaria. This may have affected the statistical analyses.

CHAPTER TWO

LITERATURE REVIEW

2.1. Biology of Nile Tilapia, *Oreochromis niloticus* Linnaeus, 1758.

2.1.1. The Taxonomy of Nile Tilapia.

Nile tilapia is classified as follows, Kingdom: Animalia, Phylum: chordata, Subphylum: vertebrata, Superclass: osteichthyes, Class: actinopterygii, Order: persiformes, Family: cichlidae, Subfamily: pseudocrenilabrinae, Tribe: tilapiini, Genus: *Oreochromis*, Species: *niloticus* (Kocher *et al.*, 1998; Nandlal and Pickering, 2004; El- Zarka, 2007; Jimmy, 2010; FAO, 2010).Tilapias are easily identified by an interrupted lateral line characteristic of the Cichlidae family of fishes (Popma and Masser, 1999; FAO, 2009).

2.1.2. Morphology of Nile Tilapia.

They are laterally compressed and are deep-bodied, with long dorsal fins. The forward portion of the dorsal fin is heavily spined. There are usually wide vertical bars down the sides of fry, fingerlings and sometimes adults (Popma and Masser, 1999) (figure 1). *Oreochromis niloticus* has strong vertical banding patterns. Mature males have gray and pink pigmentation in the throat region (Popma and Masser, 1999) and red coloration on the caudal fin (Barreto and Volpato, 2006). During spawning, pectoral, dorsal and caudal fins of *O. niloticus*, become reddish in colour while caudal fin develops numerous black bars (FAO, 2010).

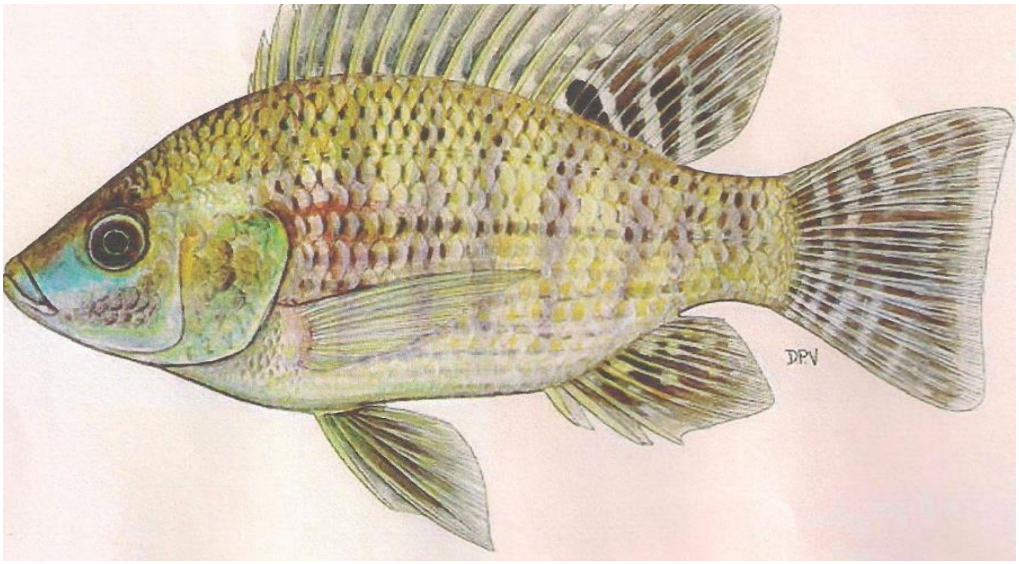

Figure 1: Nile tilapia morphology.

www.gcca.net/from/*Oreochromis_niloticus*_baring…Accessed on 20th March, 2020.

2.2: Ecology of *Oreochromis niloticus*.

Tilapia originated from Nile Valley and spread to Central and Western Africa (Nandlal and Pickering, 2004). They are among the most important warm water fishes used for aquaculture production (Charo-Karisa, 2005) and only second most popular farmed fishes after carps (El-Sayed, 2002; Getinet, 2008; Acosta and Gupta, 2009).They are recognized as one of most important species in tropical and sub-tropical aquaculture (Li *et al*., 2001; El-Sayed, 2002; Hassanien and Gilbey, 2005).

Nile tilapia was introduced into Lake Victoria in 1950s and 1960s to boost the declining fishery (Getabu, 1992; Njiru *et al*., 2006).It is now the commercially most important tilapine in Lake Victoria and third most important fishery in the lake after Nile perch (*Lates niloticus* L) and a native Cyprinid, *Rastrineobola argentea* (Pellegrin) (Njiru *et al*., 2006).Tilapia can be cultured under very basic conditions and is ideal therefore, for rural subsistence farming, yet amenable to

more sophisticated market–oriented culture programmes (Nandlal and Pickering, 2004). Nile tilapia can survive a wide range of pH (Njiru *et al*., 2006), tolerates low levels of dissolved oxygen, and feeds on a variety of food items such as higher dietary fiber and carbohydrate concentrations (Njiru *et al.,* 2006; Mjoun, 2010) among other water parameters (Table1)

Table 1: Limits and optima of water quality parameters for tilapia. Adapted from Mjourn *et al.,* 2010

Parameter	Range	Optimum for growth	Reference
Salinity, parts per thousand	Up to 36	Up 19	El-Sayed (2006)
Dissolved oxygen, mg/L	Down to 0.1	> 3	Magid and Babiker (1975); Ross (2000)
Temperature, C°	8-42	22–29	Sarig (1969); Morgan (1972); Mires (1995)
pH	3.7 -11	7–9	Ross (2000)
Ammonia, mg/L	Up to 7.1	< 0.05	El-Shafey(1998); Redner and Stickney, (1979)

2.3. Description of Stress and its Effects in Fish

The impact of a stressor is not only dependent on stressor intensity but also defined by the situation and, most importantly, by the way it is experienced by animals such as fish (Wendelaar-Bonga, 1997). Stress is commonly defined as a state of real or perceived threat to homeostasis (Smith and

Vale, 2006). It is a condition in which the dynamic equilibrium of animal`s organization is threatened as a result of the actions of intrinsic and extrinsic stimuli commonly defined as stressors (Chrousos and Gold, 1992; Wendelaar-Bonga, 1997; Schreck *et al*., 2001). Stress response is mediated by a complex and interconnected neuroendocrine, cellular and molecular infrastructure which consists the *stress system* and is located in both the central nervous system (CNS) and the periphery (Chrousos, 2009).Stress in fish involves the activation of brain pituitary-interrenal (BPI) axis and brain-sympathetic-chromaffin cell (BSCC) axis during stress with the release of corticotrophin-releasing hormone (CRH) (Abreu *et al*., 2009;Abidin *et al*., 2017), both of which are the principal messenger systems in primary stress response (Arends *et al.,* 1999).

It is reported by Barcellos *et al*., (2001) that stocking and social interactions between specific fish have significant influence on stress. Abdel-Tawwab, (2012) in a study, observed that the best growth performance of Nile tilapia was obtained when the fish were fed on the 45% CP diet and were reared at a stocking density of 150 g body mass/m^3 as opposed stocking density of 300 g body mas/m^3. Stress is considered to be a generated response but it can be modulated by specific stressor conditions. One of the most frequent causes of chronic stress is social interaction among conspecific fish (Barcellos *et al*., 2001) which causes decreased growth linked to energetic input of food and mobilization of energy reserves by physiological alterations provoked by the stress response (El-Sayed, *et al.,*2002). Mobilization of protein as an energy source has also been observed in animals subjected to chronic stress (Wendelaar-Bonga, 1997). Chronically stressed *O. niloticus* showed a reduction in approximately one-third of their final body mass index, compared with the control fish (Barcellos *et al*., 1999).

Responses to stress are broadly categorized as primary, secondary and tertiary (Cnaani *et al.*, 2004; Iwama *et al*., 2004; Biller *et al*., 2008; Abreu *et al.*, 2009) (figure 2). In addition, at cellular level

stress response is characterized by the expression of stress proteins e.g., hsp90 (85-90kDa), hsp70 (68-73kDa) and low molecular mass hsp (16-24kDa) (Iwama *et al.*, 2004). Many of these molecules are used as quantitative indicators of stress such that when fish cells face stressed condition, multifold increase in the synthesis of Hsp occurs and plays important role in combating and/or withstanding the stress (Mahanty *et al*., 2018). Physiological stress response begins as changes in tissue and organ function that attempt to cope with or compensate for the stressor, resulting in a move away from homeostasis. Such responses, include metabolic, hematological, immunological and hydromineral parameters (Cnaani *et al.*, 2004; Evans *et al*., 2008), and biochemical blood components which are indicators of fish health status (Cnaani *et al*., 2004). These changes may differ among individuals in rate or magnitude but share general characteristics in their mode of action (Schreck *et al*., 2001).

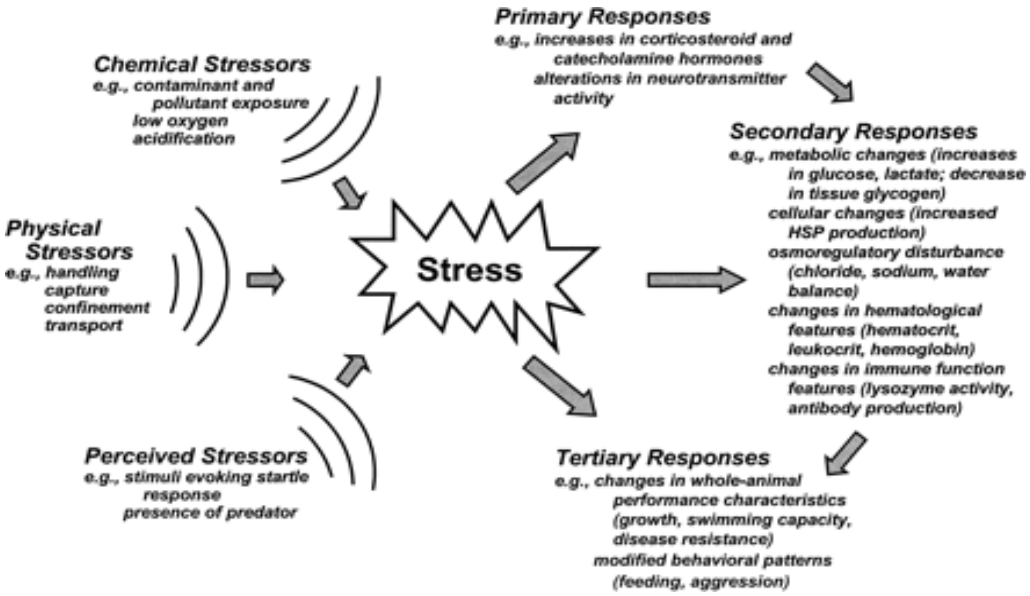

Figure 2: Physical, chemical and other fish stressors**.** Physical, chemical and other perceived stressors act on fish to evoke physiological and related effects, which are grouped as primary, secondary and tertiary or whole-animal responses. In some instances, the primary and secondary

responses in turn may directly affect secondary and tertiary responses, respectively, as indicated by the arrows. Adopted from (Barton, 2002).

2.3.1. Primary Resopnses to Stress in Fish

Primary stress responses in actinopterygian fishes include a number of hormonal changes, particularly increase in levels of circulating cortisol (Barton 2002; Karsi and Yildiz, 2005; Ndong *et al*., 2007) and catecholamines (Barton 2002; Karsi and Yildiz, 2005).Primary responses to stress are indicated by rapid elevation of plasma catecholamine and corticosteroids (Tavares-Dias *et al.*, 2001; Cnaan *et al.,* 2004; Evans *et al.,* 2008) and a partial stress response emphasizing either catecholamine or cortisol (Karsi and Yildiz 2005). The hypothalamus`s hippocampus affects the neuroendocrine stress axis through corticotrophin-releasing factor (CRF). The vasopressin, a nine amino acid peptide is an antidiuretic hormone in the kidney and acts as a neuromodulator/neurotransmitter in the brain increasing depression, responding to stressors, regulating the neuroendocrine stress axis etc. During stress, there occur signs of hyper-reactivity of the neuroendocrine stress axis (hypothalamic-pituitary – adenocortical (HPA) axis), as more ACTH and corticosterone is secreted upon stressor exposure. Hence the vasopressin that plays a critical role in ACTH secretion ensures brain centers are activated, which eventually results in the release of cortisol from the steroid-producing cells and of catecholamines from the head kidney`s chromaffin cells (Biller *et al*., 2008). These hormones singly and in combination increase glucose production (Vijayan *et al.,* 1997).Glucocorticoids i.e., cortisol, increase the availability of blood glucose to the brain, acts on the liver, skeletal muscle, white adipose tissue (WAT), and the pancreas where as in the liver, high cortisol levels increase gluconeogenesis and decrease glycogen synthesis (Figure 3) (Thau *et al*., 2020).

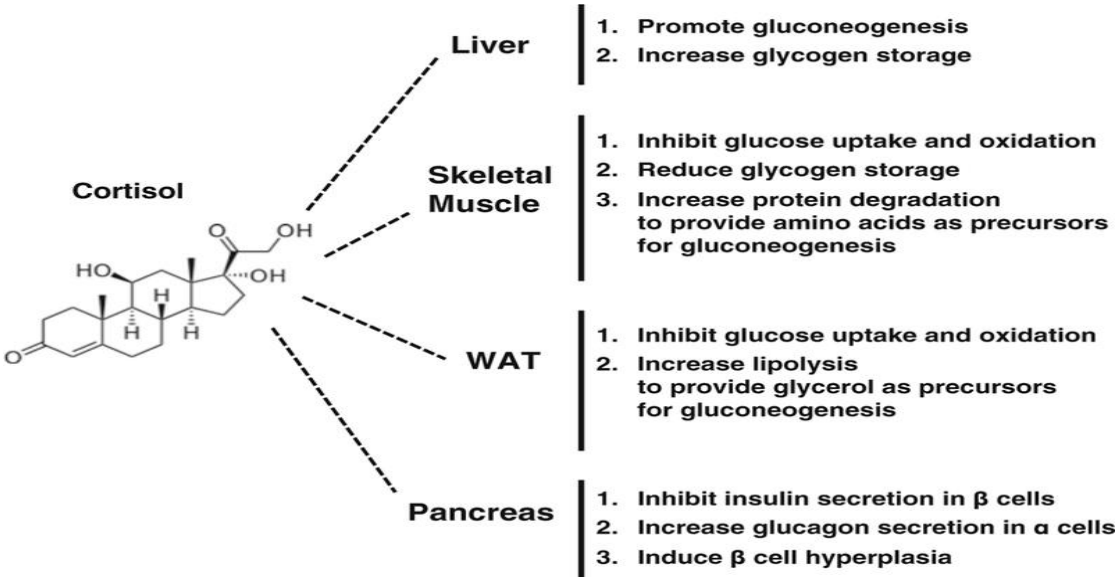

Figure 3: Glucocorticoid effects. Glucocorticoid i.e., cortisol effects on glucose homeostasis. The effects of cortisol on glucose homeostasis in peripheral tissue. (Adopted from Thau *et al*., 2020).

2.3.2. Secondary Responses to Stress in Fish

These responses comprise the various biochemical and physiological effects associated with stress, and mediated to a large extent by Catecholamines and Cortisol (Iwama *et al*., 2004). They are thus defined as the subsequent actions and effects of these hormones at blood and tissue level (Biller *et al*., 2008).Secondary responses caused by the effects of endocrine regulators, include measurable changes in blood glucose, lactate or lactic acid, major ions (e.g., chloride, Sodium and Potassium) and tissue levels of glycogen and heat shock proteins (Barton, 2002).An aspect of the secondary response consists of mobilization of energy rich substances by depletion of hepatic glycogen stores, elevation of plasma level of glucose, effects on circulating levels of fatty acids and general inhibition of protein synthesis (Schreck *et al.,* 2001).Increase in glucose concentration (Hyperglycemia) is induced by the greater glycogen depletion caused by increasing catecholamine concentration (Tavares-Dias *et al.,* 2001).

2.3.3. Tertiary Responses of Stress in Fish

This response represents whole animal and population level changes associated with stress. Chronic exposure to stressors leads to decreases in effects such as growth rate, disease resistance and reproductive success (Iwama *et al*., 2004). These responses are measured at the whole animal level, such as growth rate, metabolic rate, disease resistance, thermal and hypoxia tolerance, reproductive capacity and changes in behaviour (Barton, 2002; Cnaan, *et al*., 2004; Evans *et al*., 2008). There are differences among species (Vijayan and Moon, 1994) and among stocks of the same species in their tolerance to applied stressors (Iwama *et al*., 2004). Factors such as; genetic (e.g., species, strains), developmental (e.g., life history stage), and environmental (e.g., temperature, nutrition, water quality) influence characteristic stress response in fish (Barton, 2002).

2.4. Stress in Cultured *O. niloticus*

Nile tilapia are subjected to stress everyday with changes in culture system, water quality, environment, fish physiology and social condition constituting stress factors (Koeypuda and Jongjareanjai 2011). Stress disturbs the fine internal balance, homeostasis, and has further detrimental effects on behavior, growth, reproduction, immune function and disease tolerance (Chen*et al.,* 2004; Morales *et al.,* 2005).Different stressors cause distinct stress responses in the *O. niloticus*, thus reinforcing the specificity of stress in fish (Barreto and Volpato, 2006).

The majority of international research effort on stocing density related stress response has been done in salmonids (Ackerman *et al.,* 2000), but other species such as sea bream (*Sparus aurata* L.) (Arends *et al*., 1999) and tilapia (Barcellos *et al.,* 2001; El-Sayed, 2002; Abdel-Tawwab *et al*., 2005) have gained attention due to their potential for aquaculture in many countries. *Oreochromis niloticus* housed in aquaculture facilities or laboratory aquaria often experience overcrowding.

This subjects the fish to acute or chronic stressors, which cause decreased weight gain, decreased disease resistance and increased mortalities (Barcellos,*et al.*,2001). High stocking density affects the growth rate through stress, competition for food and living space, voluntary appetite suppression and more energy expenditure in antagonistic interactions (Bachellos andLuihier, 1999; Yi *et al*., 2004) cited in Roland *et al*., (2014) thus affecting standard length, body weight, survival, growth homogeneity, specific growth rate and feed conversion ratios (Ronald*et al*., 2014), blood glucose and cortisol concentrations, and survival (Barcellos *et al.*, 2001; El-Sayed, 2002; Abdel-Tawwab *et al*., 2005).Abou *et al.*, (2007) observed that stocking densities had negative effects on both growth and feed conversion efficiency in *O. niloticus*. In an experiment to determine the effect of stocking density on tilapia growth and survival in bamboo – net cages trial, an inverse relationship was observed between survival, growth rate and stocking density with an apparent biological and economic benefit achieved in intensive cage cultured tilapia juvenile when stocked at 150 juveniles/m^3 than 200 juveniles/ m^3 (Osofero *et al*., 2009).

The detrimental effects of high stocking density such as lower weight gain and decreased resistance to other stressors (Barcellos *et al.*,2001), aggression and cannibalism are attributed to social stressors(El-Sayed, 2002).However, several improvements in husbandry conditions, including nutrition (Abdel-Tawwab *et al*., 2005; Abou *et al*., 2007), water quality (Nandlal and Pickering, 2004) and Nile tilapia strain (Ridha, 2006), have been shown to counteract the detrimental effects of high stocking density such as compromised development and survival**.** Magnoni *et al*., (2019) in their study, summarized the output of biomarkers studied in rainbow trout exposed to a combination of chronic and acute conditions (Figure. 4) from which a clear illustration on stress associated increase plasma stress markers is indicated by positive signs (+).

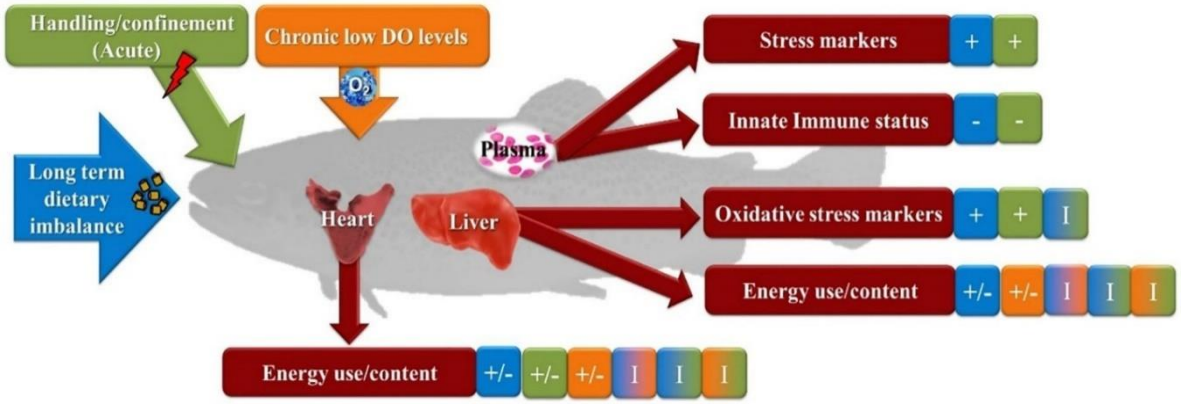

Figure 4: Summary of biomarkers in rainbow trout. Summarized output of biomarkers studied in rainbow trout exposed to a combination of chronic and acute conditions. (+) increased condition, (-) decreased condition, (I) Interactions. Source, adapted from, (Magnoni *et al.*, 2019).

2.5. Hematological, Biochemical and Cellular Markers of stress in Nile tilapia

Several haematological indices such as Red blood cell (RBC), Hemoglobin (Hb), Packed cell volume (PCV), White blood cell (WBC), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH); biochemical indices such as Plasma cortisol, Whole blood glucose (Na^+, K^+, and Ca^{++}) and Cellular indices such as heat shock proteins (i.e., Hsp 30, Hsp 60, Hsp 60) have been and still being investigated in different biological systems as biomarkers of stress. This study identified red blood cell count, plasma cortisol, whole blood glucose and blood electrolytes for investigations since they are the most studied in biological systems, more reliable in the detection of stress and the limitation in finances for the analyses of samples. Hematological and biochemical variables have become promising biomarkers in measuring the effects of aquatic pollution in fish, because blood parameters respond to low doses of pollutants and are thus considered as good physiological biomarkers in terms of anatomical, histological and endocrinal functional processes of the whole body, and therefore, they are important in diagnosing the structural and functional status of fish exposed to environmental pollutants (Seriani *et al*., 2011; Osman *et al*., 2018). These

variables are unspecific in their responses towards environmental stressors and are influenced by a variety of environmental stressors hence they have the potential of being used as biomarkers of the fish pollutions (Osman *et al*., 2018). Hematological indices have in fact been a critical component of laboratory diagnostic evaluations in many species (Old and Huveneers, 2006) like in fish following different stress conditions such as exposure to pollutants, diseases, heavy metals, hypoxia (Kefas *et al*., 2015). In a study, Ruvinda and Pathiratne (2018) observed that populations of *Oreochromis niloticus* inhabiting the polluted sites in the River Kelani were most probably experiencing stress especially due to hepatic damage and genotoxicity. The assessment of biomarker – based measure of stress is unequivocally effective diagnosis of stress (Abidin *et al*., 2017).Biomarkers thus provide important information in effect assessment studies in Nile tilapia by providing an indication on the general physiology and health status of fishes.

2.5.1. Whole Blood Cell, Erythrocytes

Blood serves as the most convenient indicator of the general condition of the animal's body (Kefas *et al*., 2015). Any change in hematological parameters could be a predictor of unfavorable environment or effect of different stress factors (Elarabary *et al*., 2017). The variation degree on the hematological response is an important tool to fish health diagnosis and may vary according to stressor (Martins *et al*., 2008). The erythrocytes or red blood cells (RBCs) and leucocytes or white blood cells (WBCs) are the most abundant cell types in the circulatory system of vertebrates (Shen *et al*., 2018). Consequently, hematological studies especially on red blood cells are promising tools for investigating physiological changes caused by environmental pollutants (Zaghloul *et al*., 2005) associated with stress. Fish erythrocytes, however, are more responsive to environmental stresses, and often vary in morphology and effectiveness of oxygen transport (Osman *et al*., 2018).

2.5.2.Plasma Cortisol

Cortisol (Figure5) is an essential hormone with a chemical formula of $C_{21}H_{30}O_5$ and molar mass of 362.460 g/mol. It has a wide variety of effects on most tissues in fish. Perry and Medbak, (2013) underscores the importance of cortisol particularly through its gluconeogenic action in coping with situations of stress such as infections.

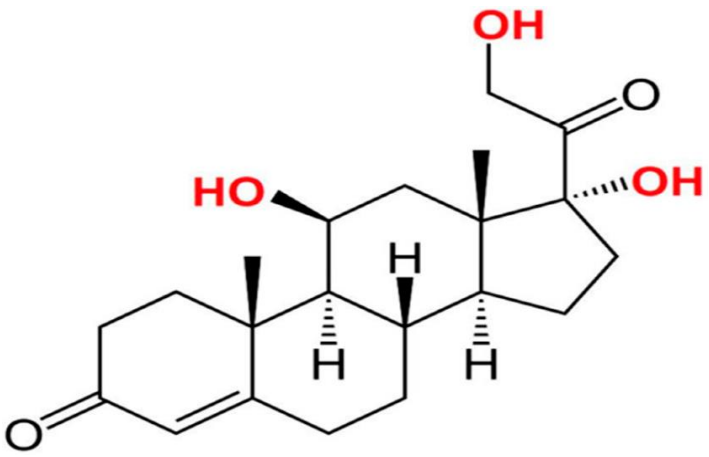

Figure 5: Chemical structure of cortisol. Chemical structure of cortisol. Source, adopted from (Abidin *et al*., 2017).

The circulating level of cortisol is used as an indicator of the degree of stress experienced by fish (Barton and Iwama, 1991; Wendelaar-Bonga, 1997) with cortisol being the principle corticosteroid in actinopterygian i.e., teleostean fishes (Barton, 2002). Kalamarz-Kubiak(2017) notes tha, the pathway for the release of cortisol initiates the activation of the Brain-Sympathetic-Chromaffin cell (BSC) axis and the (Hypothalamic-Pituitary-Internal Axis)HPI axis with the release of corticotrophin-releasing hormone (CRH), or factor (CRF). CRF which is majorly released by the para-ventricular nucleus (PVN) (Ramamoorthy 2016) of hypothalamus in the brain stimulates the corticotrophic cells of the adenohypophysis (anterior pituitary) to secrete adrenocorticotropic

(ACTH) (Abidin *et al*., 2017; Than *et al*., 2020). Circulating ACTH, in turn, stimulates the interrenal cells (adrenal cortex homologue) embedded in the kidney to synthesize and release corticosteroids into circulation for distribution by blood stream to target tissues (Figure 6**)** (Barton and Iwama, 1991; Wendelaar-Bonga, 1997).The interrenal corticosteroid cortisol is reported to affect osmoregulation, energy metabolism, immune competence (Wendelaar-Bonga, 1997) and the development processes including, hatching, metamorphosis and growth in fish (Stouthart *et al.*, 1998).

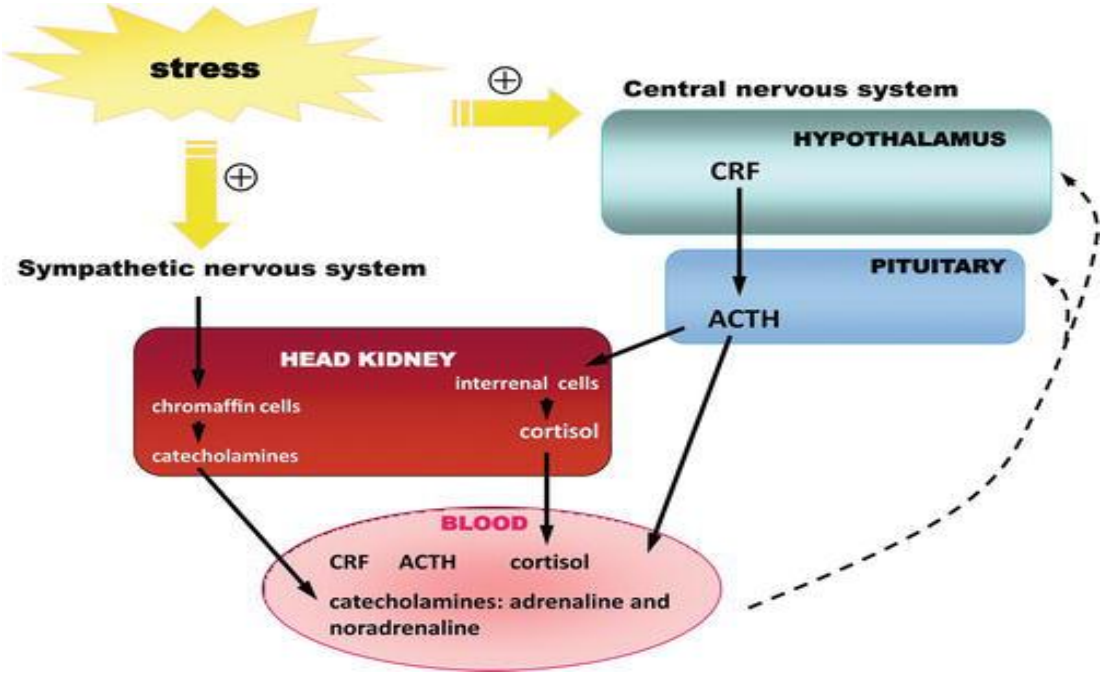

Figure 6: Stimulation of BSC axis and HPI axis. Schematic diagram on the stimulation of BSC axis and HPI axis in response to stress in fish. Adopted from (Kalamarz-Kubiak, 2017).

Cortisol causes chromaffin cells and the endings of adrenergic nerves to increase the release of catecholamines (CAs) (epinephrine, Epi and norepinephrine, NE) (Wendelaar-Bonga, 1997; Iwama *et al.*, 2004) which further increase glycogenolysis and modulate cardiovascular and

respiratory function (Reid *et al.,* 1998). In vitro exposure of hepatocytes to Epi and NE shows that CAs promote glycogenolysis through stimulation of glycogen phosphorylase, a $ß_2$-receptor-mediated process, thus this process is important when hepatic glycogen has been depleted (Wendelaar-Bonga, 1997). This whole process increases the substrate levels (glucose) from fish liver and muscle toward blood circulation, with glucose entering into cells through insulin action (Martinez-Porchas *et al*., 2009), where it plays a major role in the bioenergetics in stressed fish. Acute and chronic stress is typically associated with increased metabolic rate with plasma glucose levels being positively correlated with metabolic rate (Wendelear-Bonga, 1997). Cortisol also plays a significant role in natural regulation of ion and water balance by aiding excretion of Na^+, increasing Na^+-K^+ ATPase activity in the gills, and enhancing water absorption in the intestine and urinary bladder in teleost(Greenwell *et al*., 2003).

2.5.3. Glucose

The mechanisms of stress induced alterations in glucose metabolism in fish leading to hyperglycaemia are elucidated and involves the changes in different pathways (figure 7) with chronic stress reported to cause hyper-metabolism characterized by enhanced glycolysis, gluconeogenesis, altered glucose uptake and reduced glycogenesis (Nirupama *et al*., 2018).The elevation in blood glucose levels are reliable indicators of fish stress response (Ortuno *et al.*, 2001).Tilapia is severely glucose intolerant since it lacks insulin– sensitive glucose transporter–4(GLUT - 4) even though their islets are glucose responsive (Wright *et al*., 1998, Wright *et al*., 2015).For most fish species, cortisol is the main hormone that activates glucose, which provides an increase in the energy supply, so that fish can withstand the stress situation (Gomes,*et al.*, 2003).This explains the observations by other authors that glucose level increases, with increase in corticosteroid levels under acute stress (Rotllant and Tort, 1997; Vijayan *et al*., 1997).

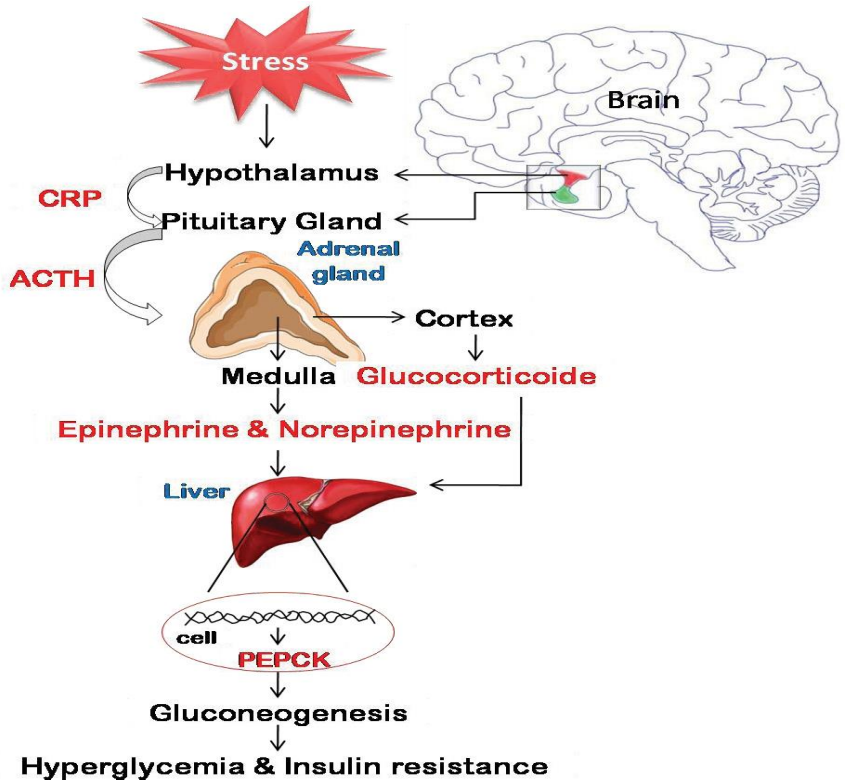

Figure 7: Hypothalamic-Pituitary-Adrenal Axis. Schematic illustration of Hypothalamic-Pituitary-Adrenal axis under chronic stress. Adopted from (Nirupama *et al.,* 2018).

Due to limited work on the effect of stocking density - induced stress on the expression of glucose in *O. niloticus*, other related studies that have used confinement, handling procedures, chemical treatment and netting induced stress are further cited to buttress the foregoing scientific assertions. Confinements stress in *O. niloticus*, was noted to increase in plasma glucose level caused by a short-term stress driven by catecholamine, whereas maintenance of higher glucose level caused by long term stress is due to the effects of cortisol (Vijayan *et al*., 1997). Rotllant and Tort,(1997) also observed that, confinement stress has led to an increase plasma glucose concentration without affecting the turnover rate in sea raven, suggesting the possibility that higher glucose concentration

in *O. niloticus* was due to enhanced production. Iwama (2002) later on noted that, handling procedures greatly increased plasma glucose concentration in fish populations exposed to environment pollutants, pathogens or poor water quality. Pasnik*et al*., (2008) observed a significant increase in mean blood glucose levels among *O. Niloticus* subjected to Tricaine Methanesulfonate (MS-222) treatment above controls following immersion in MS-222 solution, but there were no significant differences between MS 222 treated and control in blood glucose level. Li *et al.,*(2001) had earlier observed a significant increase in plasma cortisol and blood glucose levels when the fish was vaccinated with *Streptococcus agalactiae* vaccine and MS-222 anesthesia given after 30 days for the experimental fish and left in a net for 5 min. In general, chronic stress leads to higher elevation of cortisol and glucose levels in *O. niloticus* (Barcellos *et al*., 1999).

2.5.4. Blood Electrolytes

Sodium and potassium ions are some of the major monovalent ions involved in osmoregulation (Greenwell*et al*., 2003) in fish. The stress response of Nile tilapia to increased salinity was evaluated using the levels of Na^+, K^+, and Ca^{++} among other hematological indices as indicators with the secondary stress indicated by a significant decrease or increase in some biochemical variables in the blood (Karsi and Yildiz 2005).In *Micropogonias furnieri* and *Genidens barbus*, a positive relationship between plasma Na^+ and Ca^{++} levels and water salinity and a negative relationship between plasma K^+ levels and salinity was exhibited (Becker *et al*., 2011) an indication that, teleost; *Genidens barbus* and *Micropogonias furnieri* have a high capacity to regulate plasma ion levels at both low and high salinities. In a study, hypoxia stress had a direct control on brain Na^+/K^+-pump function probably due to differential activation of functional neuronal clusters in the brain segments to the challenges in oxygen delivery and Na^+ availability

(Peter and Simi 2017), this mediated by Nitric oxide (NO).NO is a major signaling and effector molecule mediating the body's response to hypoxia, given its unique characteristics of vasodilation (improving blood flow and oxygen supply) and modulation of energetic metabolism (reducing oxygen consumption and promoting utilization of alternative pathways) (Umbrello *et al*., 2013).In another study, to evaluate, if sodium binding system alleviates acute salt stress in eels, Na^+ concentration increased gradually after salt water, maximizing on day 3 and returning to a lower level in blood plasma on day 7 of the treatment (Wong *et al*., 2017). The foregoing study appropriates the proportion of the monophasic increase and decrease in Na^+ concentration to changes in Na^+ contents in the plasma, this means that under conditions that exacerbate Na^+ in the plasma, Na^+-binding molecules are produced to sequester excess ionic Na^+ to negate its osmotic potential, thus preventing acute cellular dehydration. This is consistent with the findings that, sodium concentration in blood plasma drops to 30% relative to the initial level in roach (*Rutilus rutilus*) and to 27% in bream (*Abramis brama*) during a few hours of the initial stage of stress. The transport from erythrocytes counteracts plasma hyponatremia and results in the decrease in erythrocyte sodium to 62% in roach and 61% in bream (Martemyanov, 2013).This contrasts the hypothesis that stress-induces stimulation of counter-transporting Na^+/H^+ (antiport channel) exchanger by catecholamines and consequently raising erythrocyte sodium so as to increase oxygen-carrying capacity.

2.5.5. Heat Shock Proteins

Physiological stress responses also occur at the cellular level. This is characterized by the induction of a conserved group of proteins named the heat shock proteins (Hsp) (Inoue *et al.*, 2008), classified into families based on their approximate molecular mass (e.g., Hsp90, Hsp70, Hsp60, Hsp30) (Ackerman *et al*., 2000). The expressions of these proteins in various fish tissues,

correspond to a wide range of stressors e.g. bacterial pathogens, environmental contaminants such as heavy metals, industrial effluents, pesticides and aromatic hydrocarbons, thus making these proteins to be considered as indicators of stress states in fish (Iwama, *et al.,* 2004). Hsp70 is the most extensively studied Hsp because it is the most versatile heat shock protein response to stress (Iwama *et al.,* 1999) and assists in the folding of nascent polypeptide chains. These molecular chaperones mediate the repair and degradation of altered or denatured proteins (Iwama *et al*., 2004).

Heat shock proteins (Hsp) hold a great promise as biomarkers of generalized stress in fish populations exposed to environmental pollutants, pathogens, or poor water quality. This is because the present measures of stress in fish such as increase in plasma concentrations of glucose and cortisol can be greatly influenced by stress factors such as handling procedures, while changes in tissue levels of Hsp 70 are not (Iwama *et al.,* 2004). It's therefore imperative that care is taken to avoid escalation of cortisol and glucose levels due to handling procedures. It has also been demonstrated that handling stress does not alter levels of hepatic Hsp 70 in rainbows trout (Vijayan *et al.,* 1997).

CHAPTER THREE

MATERIALS AND METHODS

3.1.Study Area

This research focuses on Nile tilapia whose fingerlings were obtained from the beaches on the Kenyan part of Lake Victoria (4,100 km^2) a fresh water lake. They were then transferred to the fish rearing facility at Maseno University (0^o 22' 11.0" S, 35^o 55' 58.0" E / Latitude; 0.369734, Longitude; 35.932779) located 400 km west of Nairobi where this study was conducted under laboratory conditions.

3.2.Experimental Procedures

The study protocol was reviewed and optimized by experts from the Department of Zoology - Maseno University, Kenya and the Department of Biological Sciences – Masinde Muliro University of Science and Technology, Kenya. Healthy Nile tilapia originally from Lake Victoria were obtained from the Department of Zoology, Maseno University`s fish rearing facility where they were reared under natural environmental conditions (natural photoperiod 12L: 12D, tropical temperature and standard water quality) and later harvested for aquaria stocking by seining technique (Gorissen and Flik 2016). The fish were first acclimatized to the conditions of the Laboratory; temperature, oxygen concentration, water favorability, water pH, nutrient consumption and utilization, photoperiod, experimental aquaria size, weight, colour and shape for 3 days and progenesis (i.e. fish obtained from the same fish holding facility) as reported in (Barton and Iwama 1991; Volpato and Fernandes 1994; Abdel-Tawwab *et al.*, 2005). Both male and female fish were randomly allocated into 2 glass indoor aquaria (0.050m^3) in triplicates at Low and High stocking densities of 150g and 300g body weight respectively. All fish were matched for body

weight (Standard mass, Mean ± SD 15 ± 1g) (Abdel-Tawwab *et al.,* 2005; Sebastiáo *et al*., 2011) and age (Barton and Iwama 1991).

The aquaria were fitted with aerator pumps (Lp Low Noise Air pumps) manufactured by Resun® - China, and mercury thermometers. Sand-fine gravel filter system was also put into each aquarium. The aquaria were filled with equal volumes (40 liters) of rain water. The fish in each aquarium were fed on a carbohydrate based feed of chick mash (18 % protein) (Sigma Feeds Ltd-Nairobi) supplemented with crushed Silver cyprinid (*Rastrineobola argentea*) to 25 % protein at a feed portion of 10 g/kg of life body weight, and the feeding was terminated 24 hours to sampling (Abdel-Tawwab *et al.*, 2005).

3.3. Sample Collection and Storage

After 21 days, the fish were anaesthetized with 2-phenoxyethanol (chemical formula -$C_8H_{10}O_2$, active substance - ethylene glycol monophenyl ether) obtained from (BDH Laboratory Reagents-England) at 0.30 ml. l^{-1}water concentration for 10 minutes before extraction of blood samples (Moreira and Volpato 2004) to minimize suffering. A large plastic sieve was then used to draw fish (n = 5) from each of the six aquaria. Blood was then drawn through the terminal procedure, cardiac/heart puncture technique using Ethylene diamine tetra acetic acid (EDTA) coated (obtained from, BDH Laboratory Reagents-England) hypodermic syringes (El-Sayed and Kawanna 2007) as an anticoagulant. This took not more than 1 min for each fish so as to eliminate any chances of rise in blood cortisol levels due to handling stress (Cnaani *et al*., 2004; Abdel-Tawwab *et al.,* 2005; Elarabary *et al*., 2017). Some of the drawn blood samples (n = 18 i.e., three samples from each of the six aquaria) were then transferred into labelled Eppendorf tubes each containing 1 ml EDTA solution, placed onto crushed ice (0^o C) in an icebox and ferried to the University`s Post Graduate laboratory at the Department of Zoology for further processing. In the

laboratory, each sample was then divided approximately into half. One portion of each sample was used for whole blood erythrocyte count while the remaining portion of the samples were subjected to centrifugation at 350 x g for 10 min at25^{o}Croom temperature to obtain plasma using a Universal 16 R centrifuge. This was done within 3 hrs. of blood extraction to avoid potassium ion leak out of the red cells (WHO, 2006). Plasma was stored at -20^{0}C for cortisol and electrolyte (sodium, potassium and calcium ions) assays.

3.4.Erythrocyte Count Assays

Erythrocyte count were determined using a customized protocol (Electron Microscopy Sciences 2020) (Appendix 1). The Natt & Herrick`s stain solution and filter was inspected for any precipitates. Whole anticoagulated blood samples were diluted using standard pipette and micropipettes with Natt & Herrick`s solution at the rate of 1:200. Diluted samples were each allowed to mix for 1 minute before discharging 10 µl of each sample into the hemocytometer. The samples` contents were each allowed 3 minutes to settle after charging the hemocytometer. Using a high dry (X40) objective lens of the microscope, total number of the RBCs that overlap the top and left border save for those that overlap the bottom or right borders, were taken for each sample in the four corners and central squares of the central large square of the improved Neubauer counting chamber. The total number of the RBCs counted for each sample was multiplied by 10,000 to obtain the total erythrocyte count per microliter (Campbell 1995).

3.5.Plasma Cortisol Concentration Level Assays

Plasma cortisol concentration (ng/ml) levels were assayed using ELISA(Enzyme-Linked Immunosorbent Assay) technique employing Neogen Corporation ELISA kit (Lansing, Ml, USA, 2010) (Evans *et al*., 2008) and the Company's Cortisol ELISA protocol(Appendix 2). This

quantitative evaluation of cortisol concentration levels in blood plasma was done at The Kenya Medical Research Institute (KEMRI) – Kisumu, Kenya. An antibody coated 96 well microplate was used. The standard solutions and the diluted samples were first added to the microplate in duplicates. Diluted enzyme conjugate was then added and the mixture shaken and incubated at room temperature for 1 hour to allow competition to take place between the enzyme conjugate and cortisol in the samples for the limited number of binding sites on the antibody coated plate. The plate was then washed with a wash buffer to remove all the unbound material. The bound enzyme conjugate was detected by the action of substrate which generated an optimal colour after 30 minutes. Quantitative test results were obtained by assaying and comparing the absorbance reading of wells of the samples against the standards with a micro plate reader set at 650 nm using SoftMax Pro Micro plate Data Acquisition & Analysis Software. The samples were each diluted ten (10) times before being assayed.

3.6.Whole Blood Glucose Concentration Level Assays

Blood glucose levels were determined using hand-held One Touch Ultra glucose meter (MD-300) from whole blood and test strips manufactured by TaiDoc. Technologies Corporation and supplied by (MD Instruments Inc.) as was established by (Karsi and Yildiz 2005; Vilisek *et al*., 2007; Inoue *et al*., 2008) at the sampling site. Whole blood was applied onto the test strips fixed in the hand-held glucose meter. Glucose concentrations were read in $mmol^{-1}$.

3.7. Blood Plasma Electrolytes Concentration Level Assays

Blood plasma electrolyte (Na^+, K^+ and Ca^{++}) concentrations were measured to compare the hydromineral balance in both the LSD and HSD *O. niloticus* groups. Flame Photometer 410 (Sherwood Scientific Ltd – UK) technique was used to analyze for Na^+, K^+ and Ca^{++}ions

concentrations in blood plasma (Viana *et al.*, 2007) in samples from control and the experimental groups of *O. niloticus* using Sherwood Scientific Ltd.'s customized protocol (Appendix5) (Sherwood Scientific LTD 2004).

Plasma samples were first filtered using Whatman Filter Papers (7-18 cm diameter). Samples were further filtered through Syringe filters (Vertical Chromatography Co., Ltd Bangkok-Thailand and supplied by Solid Phase Extraction (SPE) Filtration Products. Commercial Flame Photometer Standards (1000 ppm K, 1000 ppm Na and 1000 ppm Ca) obtained from (Sherwood Scientific Ltd-UK) were used to make calibration stock solutions by diluting and topping up 1 ml of each of the standards to 100 ml using distilled water in labeled 100 ml volumetric flasks. The stock solutions were then used to prepare working standards for sodium, potassium and calcium in clearly labeled test tubes. Each working standard solution was run one by one for these minerals and their absorbance values recorded. Each plasma samples were diluted 1: 5 with distilled water by mixing 1ml sample with 5 ml distilled water for Na^+ , K^+ and Ca^{++} concentration assays.

3.8.Statistical Analyses

Means, Standard deviations (SD) and Standard Error of Means (SEM) have been used to describe the data on both relative blood plasma cortisol, whole blood glucose, electrolyte concentrations and erythrocyte count and in LSD and HSD *Oreochromis niloticus* groups. Means and SD have been used to describe the differences in the levels of concentrations of cortisol, whole blood glucose, blood electrolytes and erythrocyte count in LSD and HSD *Oreochromis niloticus* groups. The aforementioned descriptive statistics were determined using free to use internet`s One – Way ANOVA application. Microsoft Office Professional plus Excel 2013 software was used to present the data graphically. The accuracy with which the distributions of the extracted blood samples for LSD and HSD groups represents the expression of stress related to stocking densities in

Oreochromis niloticus under aquaculture systems is measured using SD at 2 standard deviation of the means at 95 % level of confidence (Kubokawa *et al*., 1999). One - Way ANOVA was used to test the hypotheses; H_0: $\bar{x}_{LSD1} = \bar{x}_{LSD2} = \bar{x}_{LSD3}$, $\bar{x}_{HSD1} = \bar{x}_{SHD2} = \bar{x}_{HSD3}$, H_A: not H_0 at F_{crit} (df_{AMONG} = 2, df_{WITHIN} = 6, α = 0.05) = 5.14 for individual samples and H_0: $\bar{x}_{LSD1,2,3} = \bar{x}_{HSD1,2,3}$, H_A: not H_0 for the true sample means at F_{crit} (df_{AMONG} = 1, df_{WITHIN} = 4, α = 0.05) = 7.71 for cortisol, F_{crit} (df_{AMONG} = 2, df_{WITHIN} = 12, α = 0.05) = 3.89 for whole blood concentration for LSD_1, LSD_2, LSD_3 and HSD_1, HSD_2, HSD_3 individual sample groups. Similar statistical analyses were done on plasma electrolyte true sample mean concentrations, F_{crit} (df_{AMONG} = 1, df_{WITHIN} = 4, α = 0.05) = 7.71 for N^+, K^+ and ca^{++} concentrations for $LSD_{1, 2, 3;}$ $HSD_{1, 2, 3}$ groups and lastly for red blood cell counts at F_{crit} (df_{AMONG} = 1, df_{WITHIN} =18, α = 0.05) = 4.41 for LSD and HSD sample groups.

CHAPTER FOUR

RESULTS

4.1. Erythrocyte Count

One-Way ANOVA analysis revealed statistical ($P < 0.05$) difference in the means of Erythrocyte counts (Table 2) in LSD and HSD O. *niloticus* groups ($F_{(df, 1; 18)} = 136.06 > F_{crit.} = 4.41$; P = 0.00). Mean Erythrocyte count was statistically ($P < 0.05$) higher for HSD than LSD O. *niloticus* groups at mean ± SD, 7.01 ± 0.77 mm^3 and 3.36 ± 0.63 mm^3 respectively (Table 2).

Table 2: Erythrocyte count Means ± SD for LSD and HSD groups,

Statistics	Erythrocyte Mean ± SD count x 10^6 per mm^3	
	LSD	HSD
Mean	3.36	7.01
SD	± 0.63	± 0.77

4.2 Plasma Cortisol

To establish the concentration of plasma cortisol, a cascade of parameters were determined following an established sequence by first obtaining Optical Density (Absorbance Values) and the percent maximal binding ($\%B/B_o$ values)(Appendix 3) which was used to create a standard curve graph employed to determine the samples` concentrations from their respective $\%B/B_o$ and then multiplied with a dilution factor of 10 to give plasma cortisol levels and means x 10 ng/ml for LSD and HSD groups at ($P < 0.05$) (Figure 9). These readings were thus used to determine the working mean values. The $\%B/B_0$ values were determined by dividing the averages of each standard absorbance value (B_1 ….. B_7) by the B_0 absorbance value and then multiplied by 100 to achieve the percentages**.** The data from (Appendix 3)was used to obtain the standard curve (Figure 8)by

plotting the $\%B/B_0$ for each standard concentration on the ordinate (y) axis against concentration on the abscissa (x) axis a using a curve-fitting routine.

One-Way ANOVA analysis revealed a statistical ($P< 0.05$) difference in the means of plasma cortisol concentrations in HSD and LSD *O. niloticus* groups ($F_{(df,\ 1;\ 18)} = 7.946 > F_{crit.} = 4.414$; $P = 0.01$). Mean plasma cortisol concentration was statistically ($P \leq 0.01$) higher for HSD than LSD *O. niloticus* groups at mean ± SD, 6.35 ± 0.89 ng/ml and 4.49 ± 1.08 ng/ml respectively (Table 4). It is evident from the small values of Standard error, SE, (0.33_{LSD}; 0.50_{HSD}) that the sample means (4.62_{LSD}; 6.35_{HSD}) are reliable indications of cortisol levels in *O. niloticus* reared under the two stocking densities in aquaculture systems.

One-Way ANOVA analysis of the triplicate *O. niloticus* control groups gave no statistical ($P > 0.05$) difference in their means ($F_{(df,\ 2;\ 6)} = 0.26 < F_{crit.} = 5.14$; $P = 0.78$) at 4.80 ± 0.53, 4.80 ± 1.12 and 3.87 ± 2.83 mg/dl for LSD_1, LSD_2 and LSD_3 respectively. Similarly, no statistical ($P > 0.05$) difference ($F_{(df,\ 2;\ 6)} = 0.43 < F_{crit.} = 5.14$; $P = 0.67$) was revealed between the means of the triplicate *O. niloticus* experimental groups at 6.63 ± 2.12, 6.60 ± 0.64 and 5.83 ± 1.34 mg/dl for HSD_1, HSD_2 andHSD_3 respectively using One-Way ANOVA analysis. One-Way ANOVA analysis between the means4.49 ± 1.08 and 6.35 ± 0.89 for $LSD_{1,2,3}$ and $HSD_{1,2,3}$ groups respectively revealed significant statistical($p < 0.05$) difference ($F_{(df,\ 2;\ 6)} = 20.32 > F_{crit.} = 5.14$; $P = 0.01$) between the two stocking densities. The small values of SEM (0.31_{LSD}; 0.27_{HSD}) for the true means (4.49_{LSD}; 6.35_{HSD}) confirm the fact that, the true means vary negligibly from the control and experimental groups' means, and are therefore reliable. This is shown on (Table 3), which gives both between and within group means and their SD for LSD and HSD groups for purposes of comparison of Plasma Cortisol Levels and means at $P < 0.05$.

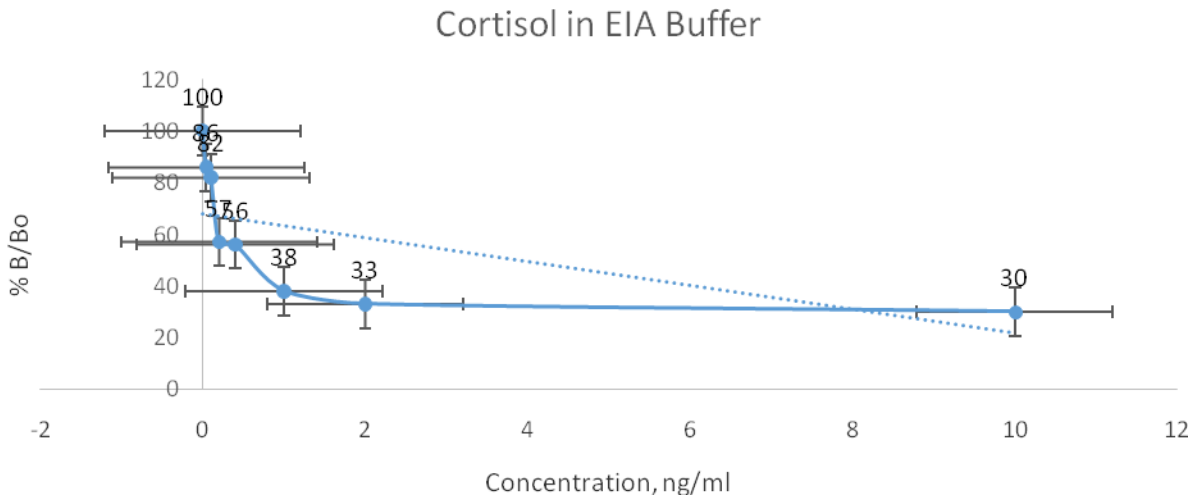

Figure 8: Standard Curve for cortisol ELISA. It is used to compare %B/B$_o$ of each sample to the corresponding concentration of cortisol standards to determine the concentration of cortisol in each sample.

Table 3: Plasma Cortisol Concentrations, Means Within and Between Groups, SD, SE and SEM for LSD and HSD Triplicates at *P<0.05,* sample size (n = 18)

Sample Number	**Cortisol Concentrations, x 10 in ngml^{-1}**							
	LSD$_1$	**LSD$_2$**	**LSD$_3$**	**Mean**	**HSD$_1$**	**HSD$_2$**	**HSD$_3$**	**Mean**
1	5.2	3.5	5.6	4.8	5.6	6.7	4.28	5.5
2	4.2	5.6	0.6	3.5	8.3	7.2	6.7	7.4
3	5.0	5.2	5.4	5.2	6.0	5.9	6.5	6.1
Mean	**4.80**	**4.80**	**3.87**	**4.49***	**6.63**	**6.60**	**5.83**	**6.35***
SD	**0.53**	**1.12**	**2.44**	**0.89**	**2.12**	**0.64**	**1.34**	**1.08**
SE	**0.31**	**0.65**	**1.41**	**-**	**1.22**	**0.37**	**0.77**	**-**
SEM	**-**	**-**	**-**	**0.51**	**-**	**-**	**-**	**0.62**

Note that, the means number marked with asterisk are the mean of means computed LSD$_2$ and HSD$_2$ group means.

Table 4: summary of Plasma Cortisol Concentrations Means, SD, SE and SEM for HSD and LSD *O. niloticus* at $P < 0.05$,

Statistics	Plasma Cortisol Mean ± SD x 10 ng/ml	
	HSD	LSD
Mean	6.35	4.49
SD	± 0.89	± 1.08
SE	0.33	0.50
SEM	0.51	0.62

4.3 Whole Blood Glucose

One-Way ANOVA analysis of the triplicate *O. niloticus* control groups gave no statistical ($P< 0.05$) difference in their means ($F_{(df, 2; 12)} = 0.161 < F_{crit.} = 3.885$; $P \geq 0.01$) at 77.76 ± 12.03, 74.52 ± 11.64 and 78.12 ± 9.23 mg/dl for LSD_1, LSD_2 and LSD_3 respectively. Similarly, no statistical ($P< 0.05$) difference ($F_{(df, 2; 12)} = 0.674 < F_{crit.} = 3.885$; $P \geq 0.01$) was revealed between the means of the triplicate *O. niloticus* experimental groups at 100.8 ± 14.40, 94.68 ± 5.78 and 95.04 ± 4.67 mg/dl for HSD_1, HSD_2andHSD_3 respectively using One-Way ANOVA analysis (Table 5 and 6). One-Way ANOVA analysis revealed statistical ($P< 0.05$) difference in the means of whole blood glucose concentrations in HSD and LSD *O. niloticus* groups ($F_{(df, 1; 8)} = 31.845 > F_{crit.} = 5.318$; $P = 0.01$).

Mean plasma glucose concentration was statistically ($P \leq 0.01$) higher for HSD than LSD *O. niloticus* groups at mean ± SD, 96.84 ± 5.28 mg/dl and 76.80 ± 5.92 mg/dl respectively. Like in cortisol, the small values of SEM (2.65_{LSD}; 2.36_{HSD}) for the true means (76.80 ± 5.92 $_{LSD}$; 96.84 ± 5.28 $_{HSD}$) similarly, confirm the fact that, the true means vary negligibly from the control and experimental groups' means, and are therefore reliable (Table6).

Table 5: Whole Blood Glucose concentrations, Means Within and Between Groups, SD, SE and SEM for LSD and HSD Triplicates at *P<0.05*, Sample size (n = 30).

Sample	Concentrations ($mmol \cdot l^{-1}$ x 18) in $mgdl^{-1}$							
Number	LSD_1	LSD_2	LSD_3	Mean	HSD_1	HSD_2	HSD_3	Mean
1	84.6	79.2	66.6	76.8	99.0	90.0	99.0	96.0
2	57.6	77.4	88.2	74.4	126.0	95.4	93.6	105.0
3	84.6	79.2	72.0	78.6	91.8	102.6	90.0	94.8
4	86.4	82.8	86.4	85.2	91.8	88.2	91.8	90.6
5	75.6	54.0	77.4	69.0	95.4	97.2	100.8	97.8
Mean	**77.76**	**74.52**	**78.12**	**76.80***	**100.80**	**94.68**	**95.04**	**96.84***
SD	**12.03**	**11.64**	**9.23**	**5.92**	**14.40**	**5.78**	**4.67**	**5.28**
SE	**5.38**	**5.21**	**4.13**	**-**	**6.44**	**2.58**	**2.09**	**-**
SEM	**-**	**-**	**-**	**2.65**	**-**	**-**	**-**	**2.36**

Note that, the means marked with asterisk are the mean of means for LSD and HSD groups` glucose concentrations.

Table 6: Sumarry of Whole Blood Glucose Concentration Group Means, SD, SE and SEM for LSD and HSD at *p<0.05*.

Sample	Concentrations ($mmol \cdot l^{-1}$ x 18) in $mgdl^{-1}$	
No.	LSD	HSD
Mean	76.8	96.84
SD	5.92	5.28
SE	2.53	3.23
SEM	2.65	2.36

4.4 Plasma Sodium, Potassium and Calcium Ions

Absorbance measurements for the working standards were taken (Appendix 4) and used to constructed standard curves (calibration charts) for the ions by plotting the absorbance values against the working standards concentrations (Akhtar *et al*., 2009). The graphs were individualized for the ions since each element has its own characteristic curve thus separate calibration curves had to be constructed (Sherwood Scientific Ltd.'s protocol).The concentrations of respective ions

in the samples were then determined graphically from their individualized standard curves (calibration charts) drawn using a curve-fitting routine (Figures 9, 10 and 11). The individual sample`s element absorbance values were thus read against concentrations from the calibration charts for Na^+, K^+ and Ca^{++} ions respectively. The resulting concentrations were then multiplied with a dilution factor of 5 and then converted to mmol/l for Na^+, K^+ and Ca^{++} ion concentrations (Tables 7 and 8). One-Way ANOVA analysis was carried out to check for any significance differences between the mean electrolytes concentrations in the triplicate control groups (LSD_1, LSD_2 and LSD_3) and experimental groups (HSD_1, HSD_2 and HSD_3) for each one of the three electrolytes. In the LSD groups, plasma sodium, potassium and calcium ion concentration means ± SD (**Na^+**, 1.24 ± 0.13, 1.10 ± 0.10, 0.99 ± 0.17; **K^+**, 0.41 ± 0.90, 0.41 ± 0.04, 0.44 ± **NaN**; **Ca^{++}**, 0.13 ± 0.00, 0.13 ± 0.00, 0.13 ± 0.00 mml/l) were not significantly ($P > 0.05$) different ($F_{(df, 2; 6)}$ = 0.147 <$F_{crit.}$ = 5.14; P = 0.867, $F_{(df, 2; 6)}$ = 1.244 <$F_{crit.}$ = 5.14; P = 0.350 and $F_{(df, 2; 6)}$ = NaN <$F_{crit.}$ = 5.14; P = NaN) for each of the three electrolytes from the triplicate control groups respectively. Similarly, in the HSD groups, plasma sodium, potassium and calcium ion concentration means ± SD (**Na^+**, 1.68 ± 0.23, 1.57 ± 0.22, 1.64 ± 0.32; **K^+**, 0.69 ± 0.10, 0.71 ± 0.07, 0.80 ± 0.09; **Ca^{++},** 0.13 ± 0.00, 0.13 ± 0.00, 0.13 ± 0.00 mml/l) were not significantly ($P< 0.05$) different ($F_{(df, 2; 6)}$ = 0.147 <$F_{crit.}$ = 5.14; P = 0.867, $F_{(df, 2; 6)}$ = 1.244 <$F_{crit.}$ = 5.14; P = 0.350 and $F_{(df, 2; 6)}$ = NaN <$F_{crit.}$ = 5.14; P = NaN) for each of the three electrolytes from the triplicate control groups respectively. The established no significant differences between specific plasma ions mean concentrations from samples from both the LSD and HSD groups were thus due to the standardization of the experimental conditions therefore, providing perfect average basal mean values for comparisons for any significant elevations in the individual electrolyte concentrations in the experimental fish against the control *O. niloticus* groups, however this may have no relevance in respect of Ca^{++}ions

since all the samples recorded a constant Flame photometer reading of 0.13 mmol/l. This reading forms the basis on which the statistical analysis of Ca^{++} means returned undefined *F* and *P* values of NaN. One-Way ANOVA analysis revealed that, plasma Na^{+} concentration mean ± SD (1.63 ± 0.18 mmol/l) from HSD_1, HSD_2, HSD_3 groups means was significantly ($P< 0.05$) higher than the mean ± SD (1.11 ± 0.08 mmol/l) from LSD_1, LSD_2, LSD_3 ($F_{(df,\ 1;\ 4)} = 21.799 > F_{crit.} = 6.94$, $P = 0.010$). Similar analysis on plasma K^{+} ion concentration means returned significantly ($P< 0.05$) higher mean ± SD (0.73 ± 0.03 mmol/l) from HSD reared *O. niloticu*s than LSD mean ± SD (0.42 ± 0.02 mmol/l) ($F_{(df,\ 1;\ 4)} = 345.986 > F_{crit.} = 6.94$, $P = 0.000$). Lastly, the plasma Ca^{++} ion concentration means ± SD (0.13 ± 0.00 and 0.13 ± 0.00) respectively for both HSD and LSD obtained from One-Way ANOVA analysis of within groups means returned no significant ($P< 0.05$) difference (, $F_{(df,\ 1;\ 4)} = NaN > F_{crit.} = 6.94$, $P = NaN$) but undefined. Table 9: gives a summary of plasma electrolyte mean concentrations in mmol/l, SD, SE and SEM for HSD and LSD *O. niloticus* Groups while Table 10, gives a summary on the average ± SD and *F*-test values for all Hematological and biochemical parameters evaluated in this study.

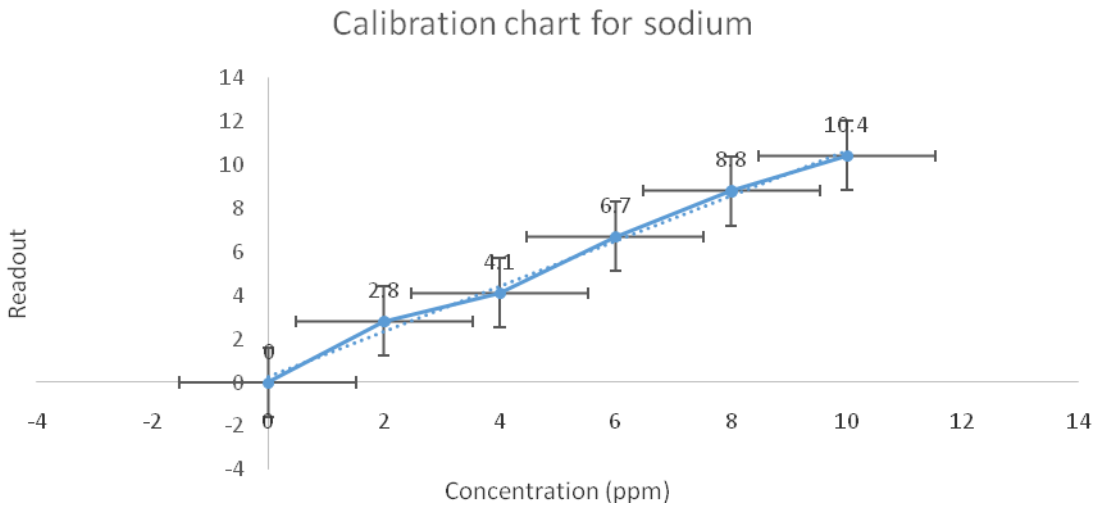

Figure 9: Standard Curve for Sodium. Compares the scale reading for sample solution with the curve to obtain the concentration of sodium in ppm.

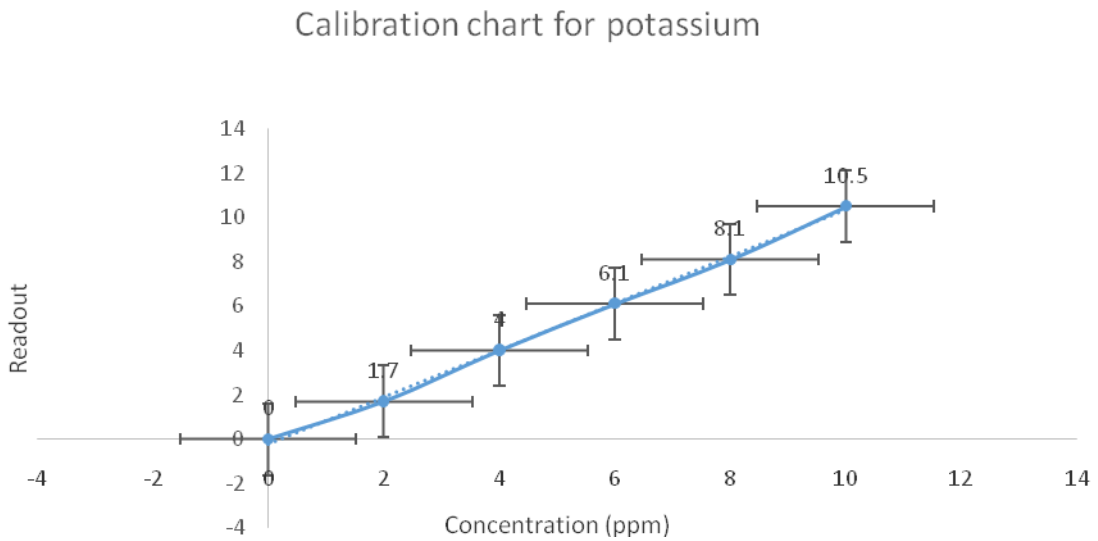

Figure 10: Standard Curve for Potassium. Compares the scale reading for sample solution with the curve to obtain the concentration of potassium in ppm

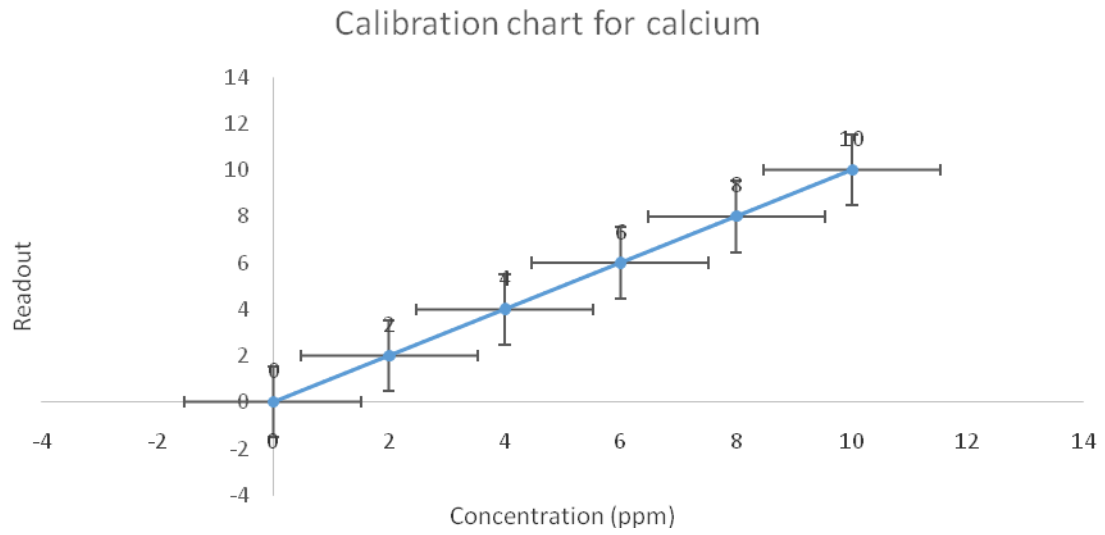

Figure 11: Standard Curve for Calcium. Compares the scale reading for sample solution with the curve to obtain the concentration of calcium in ppm.

Table 7.Plasma Electrolyte (Na+, K+ and Ca+) Concentrations in mmol/l for LSD *O. niloticus* Groups in Triplicate at *P>0.05,* Sample size (n = 9) for each electrolyte.

Sample No.	Concentration (ppm x 5 dilution factor x mmol/l)											
	Na^+ (ppm x 0.0435 mmol/l)			K^+ (ppm x 0.0256 mmol/l)			Ca^{++} (ppm x 0.0250 mmol/l)			Mean		
	1	2	3	1	2	3	1	2	3	Na^+	K^+	Ca^{++}
1	1.09	1.20	0.80	0.32	0.45	0.53	0.13	0.13	0.13	1.03	0.43	0.13
2	1.31	1.11	1.11	0.40	0.40	0.40	0.13	0.13	0.13	1.18	0.40	0.13
3	1.33	1.00	1.07	0.50	0.37	0.38	0.13	0.13	0.13	1.13	0.42	0.13
Mean	1.24	1.10	0.99	0.41	0.41	0.44	0.13	0.13	0.13	1.11*	0.42*	0.13*
SD±	.13	.10	.17	.09	.04	NaN	.00	.00	.00	.08	.02	.00

Note that, the means marked with asterisk are the Mean of means for N^+, K^+ and Ca^{++}concentrationsfor LSD group.

Table 8: Plasma Electrolyte (Na+, K+ and Ca+) Concentrations in mmol/l for HSD *O. niloticus* Groups in Triplicates at $P < 0.05$ Sample size (n = 9), for each electrolyte.

Sample No.	**Concentration (ppm x 5 dilution factor x mmol/l)**											
	Na^+ (ppm x 0.0435 mmol/l)			**K^+ (ppm x 0.0256 mmol/l)**			**Ca^{++} (ppm x 0.0250 mmol/l)**			**Mean**		
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**Na^+**	**K^+**	**Ca^{++}**
1	1.70	1.57	2.00	0.68	0.74	0.76	0.13	0.13	0.13	1.76	0.73	0.13
2	1.44	1.35	1.50	0.60	0.63	0.90	0.13	0.13	0.13	1.43	0.71	0.13
3	1.90	1.78	1.41	0.79	0.77	0.73	0.13	0.13	0.13	1.70	0.76	0.13
Mean	1.68	1.57	1.64	0.69	0.71	0.80	0.13	0.13	0.13	1.63*	0.73*	0.13*
SD±	.23	.22	.32	.10	.07	.09	.00	.00	.00	.18	.03	.00

Note that, the means marked with asterisk are the Mean of means for N^+, K^+ and Ca^{++}concentrations for HSD group.

Table 9: Summary of Plasma Electrolyte Mean Concentrations in mmol/l and SD at $p < 0.05$ and $p > 0.05$ for HSD and LSD O. niloticus Groups.

Statistics	**HSD**			**LSD**		
	Na+	**K+**	**Ca++**	**Na+**	**K+**	**Ca++**
Mean	1.63	0.73	0.13	1.11	0.42	0.13
SD	± 0.18	± 0.03	± 0.00	± 0.08	± 0.02	± 0.00

Table 10: Summary of Average Values +/- Standard Deviation of Biochemical and Hematological Parameters of *O. niloticus* Subjected to LSD and HSD/ Chronic stress.

Blood parameters	LSD	HSD	F-test
Glucose (mg/dl)	76.80 ± 5.92	96.84 ± 5.28	20.32**
Cortisol (ng/ml)	4.49 ± 1.08	6.35 ± 0.89	7.45**
Na^{+}(mmol/l)	1.11± 0.08	1.63 ± 0.18	21.80**
K^{+}(mmol/l)	0.42 ± 0.02	0.73 ± 0.03	345.99**
Ca^{++} (mmol/l)	0.13 ± 0.00	0.13 ± 0.00	NaN*
Erythrocyte count (mm^{-3})	3.36 ± 0.63	7.01 ± 0.77	136.06**

- Significant at 0.05 **; - Undefined at 0.05 *.

CHAPTER FIVE

DISCUSION

5.1. Introduction

This study sought to evaluate the hematological and biochemical indices as alternative markers of stocking density induced stress in cultured Nile tilapia subjected to stocking density induced stress.

5.2. Relative Hematological Parameter; Erythrocyte Count

Erythrocyte counts are indicators of the fish`s body general status under stress conditions. Erythrocyte count for HSD and LSD *O. niloticus* groups realized in this study compares favorably with erythrocytes count at 6.93×10^6 mm^{-3} for *O. niloticus* under culture in semi-intensive system (Bittencourt *et al*., 2003). However, in a study by Osman *et al*., (2018), there was , significant reductions in the values of RBCs, in the blood of Nile tilapia and African catfish collected from downstream sites (polluted sites/pollution stress) compared to those collected from less polluted upstream sites of River Nile(Osman *et al*., 2018).The reduced erythrocyte parameter is hypothesized to be an indication of macrocyticanemia emanating from increased destruction and subsequent enhanced erythropoiesis in the liver (Kefas *et al*., 2015) leading to low RBCs count. This is as a result of pollution stress that triggered production of cortisol (Bittencourt *et al*., 2003; Abdel-Tawab 2012) leading to macrocytic anemia and erythropoiesis. This is supported by a study by Falcon*et al*., (2007) that reported elevated levels of cortisol that in turn lead to increased erythropoietin concentration stimulating erythrocytes production thus increase in the concentration of erythrocyte which translates into enhanced oxygen loading efficiency and supply to tissue cells thus the significant elevation in the erythrocyte count in this study. As stress levels rises, there is increased demands by an organism for oxygen that is compensated by the additional introduction

of more erythrocytes from depot to blood (Martemyanov 2013) for more oxygen loading for food substrate's oxidation to release energy the fish uses to cope with the stress (Mommsen *et al*., 1999) further supporting increased erythrocyte that characterized this study. Increased in erythrocyte count can therefore be used as biomarker for stress in Nile Tilapia under culture production.

5.3. Relative Biochemical Parameters; Plasma cortisol, Whole Blood Glucose and Blood Erythrocyte counts.

5.3.1 Plasma cortisol

Plasma cortisol values obtained from HSD and LSD groups (Table 2)falls within the reported normal mean basal cortisol range of 5 – 60 ng/ml for *O. niloticus* (Cnaani *et al*., 2004; Evans *et al*., 2004). This range also accommodates the mean ± SD basal value range of 31.08 ± 4.94 to 47.97 ± 9.37 ng/ml (n = 6) obtained on day 0 in an experiment on conditioning of stress in Nile tilapia (Barton and Iwama, 1991). The findings compare well with a study of Trout subjected to an acute stressor where, higher plasma cortisol levels as well as increased levels of metabolic markers associated with oxidative stress in the liver (Magnoni *et al*., 2019). In addition, a study by Evans *et al*., (2004), reported a mean basal plasma cortisol ranges of; 5 – 15 ng/ml, 16.43 – 39.22 ng/ml and 5 – 50 ng/ml for *O. niloticus*, and 20 – 60 ng/ml for a related Cichlid, *O. mossambicus.* This study`s findings of 6.35 ± 1.06 ng/ml and 4.49 ±1.08ng/ml falls within the foregoing ranges. Studies involving other fish families such as Cyprinid *Cyprinus carpio*, Salmonids *Oncorhynchus clarkii*, and *Oncorhynchus mykiss* showed similar ranges of basal plasma cortisol levels under varied experimental conditions. Carneiro and Urbinati (2001) observed increased levels of plasma cortisol in matrinxä, *Brycon cephalus* subjected to transport operations induced stress. However, the relatively low concentrations of cortisol in both the HSD and LSD groups of *O. niloticus* may have been as a result of extrinsic nature where response is affected by external factors such as

season, time of the day and from intrinsic nature dependent on the genotype or phenotype of the fish such as rapid conversion of cortisol into less immune reactive cortisone as was observed in a related study by (Volpato and Fernandes 1994). It should also be noted that, differences in corticosteroid stress responses exist among stocks of the same fish hence the low cortisol levels recorded (Barton 2002).

Effects of extrinsic stress factor(s) in HSD and LSD *O. niloticus* groups could have compromised plasma cortisol release because the interrenal tissues may have become less sensitive to the action of ACTH or other pituitary hormones leading to low cortisol secretion (Volpato and Fernandes 1994). Different hormones such as alpha-melanocyte stimulating hormone (MSH), endorphin from the pars intermedia (PI) and some sympathetic nerve fibers (Pasnik *et al*., 2008) have been implicated in cortisol release during the chronic phase in fishes, functioning as an emergency system. However, if the sub-optimal condition persists, this system may be deleted (Abou *et al*., 2007) leading to impaired cortisol release in fish subjected to stressors. It should however be noted that, the net effect of these apparent unknown stress factors had no bearing on cortisol levels of the control and experimental fish because of the standardized experimental conditions in this study since it's a fact of science chronic sub-optimal rearing conditions may also give rise to stress responses if full adaptation is not displayed (Magnoni e*t al*., 2019).Both physiological and biological status of the fish used in this study were standardized prior to the experiment leading to a basal cortisol mean value of 4.49 ± 1.08 ng/ml in LSD reared fish against which the rise in plasma cortisol in HSD reared *O. niloticus* is compared. In the current study, the high plasma cortisol concentration level in HSD group than in LSD group was stress-induced as was similarly observed in a related study by (Douglas 2005), with chronic stress (i.e. high stocking density) producing a sustained elevation of cortisol in fish (Volpato and Barreto 2001; Barreto and Volpato 2006).

Elevated plasma cortisol therefore indicates that chronic stress occurred in HSD reared *O. niloticus* (Volpato and Fernandes 1994; Abou *et al*., 2007). Plasma cortisol is actually a good acute stress marker (Arends 1999), with adrenaline considered as the stress hormone, and cortisol the adaptive hormone (Biswas *et al*., 2004).

5.3.2. Whole Blood Glucose

Since glucose is an innate immune parameter mediated by stress, increase in glucose concentration is a secondary response to stress and the level of the increase is a measure of stress (Goncalves-de-Freitas and Mariguela 2006). Glucose concentration has been widely used in a variety of fish species, as an indicator of stress in genetic studies (Volpato and Fernandes 1994; Martinez-Porchas *et al*., 2009).

A significant ($P< 0.05$) difference in the true means of whole blood glucose concentrations in HSD and LSD *O. niloticus* groups at mean ± SD, 96.84 ± 5.28 mg/dl and 76.80 ± 5.92 mg/dl respectively was established in this study. The low values of SEM (2.65_{LSD}; 2.36_{HSD}) for the true means (76.80 ± 5.92 $_{LSD}$; 96.84 ± 5.28 $_{HSD}$) similarly, reinforces the fact that, the true means of the control and experimental groups don't vary significantly, and are therefore reliable (Table 5) thus attributable to the differences in treatments.

Glucose is an indicator of sympathetic activation under stress conditions (Pasnik *et al*., 2008). Cnaani *et al.,* (2004) reports a mean basal glucose concentration of 39.6 and 34.2 mg/dl for electroshock and social stressors respectively both of which induced acute stress in *O. niloticus*. These concentrations compare favorably with whole blood glucose mean concentration of 76.82 ± 5.92 mg/dl obtained from the triplicate LSD control *O. niloticus* groups. In addition, the triplicate *O. niloticus* groups' whole blood concentration means for LSD_1, LSD_2 and LSD_3 respectively showed no statistical difference. Similarly, no statistical difference was established between the

means of the triplicate *O. niloticus* experimental groups; HSD_1, HSD_2andHSD_3 respectively (Table 5). This is linkable to the standardized experimental conditions. For this study, mean ± SD for blood glucose concentration level was statistically high in HSD at 96.84 ± 5.28 mg/dl than in LSD at 76.80 ± 5.92 mg/dl for the *O. niloticus* groups. This is supported by strong evidence that, the blood glucose concentration means for both experimental and control groups were statistically ($P< 0.05$) different thus proving that high stocking density induced stress in *O. niloticus*. Stress hormones; adrenaline and noradrenaline in jointly with cortisol mobilized together with elevated glucose concentration levels to cope with the energy requirements as a response to stress induced by high stocking density, hence increase in blood plasma glucose level in HSD *O. niloticus*. Whole blood glucose concentration has been used as an indicator of environmental stress to reflect changes in carbohydrate metabolism under stress conditions (Kamal and Omar, 2011) a phenomenon reported by this study.

The high whole blood glucose concentration means ± SD (96.84 ± 5.28 mg/dl) recorded for HSD in this study can be associated with the single independent variable introduced, high stocking density thus indicating the occurrence of chronic stress in experimental *O. niloticus* group, since any stress condition carries additional energetic requirements (Magnoni *et al*., 2019). This shows consistency with earlier studies which have reported statistically increased blood glucose levels in stressed fish. Carneiro and Urbinati (2001) observed increased levels of glucose in matrinxä, *Brycon cephalus* subjected to hauling operations induced stress. In a study, the plasma glucose levels in the tilapia exposed to 18 ppt salinity for 72 hours were relatively high when compared to control and the fish exposed to 9 ppt salinity (Karsi and Yildiz 2005).This is validated by a significant high concentration levels of glucose in the blood of *Clarias gariepinus* and *Oreochromis niloticus* collected from downstream of the Nile river (polluted area) compared to

the river`s upstream sites (Osman *et al*., 2018). A three week duration of crowding induced stress elevated the concentration glucose levels in *Sparus auratus* (Volpato and Fernandes 1994). During stress episodes, catecholamine act precisely on the liver to trigger glycogenolysis, which leads to marshaling of glucose (Rotlant and Tort 1997). Catecholamines stimulate the enzyme glycogen phosphorylase`s phosphorylation resulting in enhanced glycogenolysis. Increase in glycogenolysis and/or a reduction in glucose clearance from the blood is what leads to the elevation in the concentration of plasma glucose in *Oreochromis niloticus* experiencing stress (Herrera *et al*., 2019).

5.3.3. Plasma Sodium, Potassium and Calcium Ions

The kidney principally retains and excretes electrolytes and fluid in healthy fish (Bockenkamp *et al*., 2003) with physiological stress being one of the factors that play significant role in the regulation of fluid and electrolyte balance in organisms i.e., fish (Balci *et al*., 2013). The current study, showed significant ($P< 0.05$) variations in the means for Na^+ ion concentrations recorded with the mean ± SD, for HSD reared *O. niloticus* group significantly ($P = 0.010$) higher at 1.63 ± 0.18 mmol/l than that for LSD at 1.11 ± 0.08 mmol/l. A similar trend in findings occurred in which, a significant ($P< 0.05$) variation in the means for K^+ ion concentrations was recorded with the mean ± SD, for HSD reared *O. niloticus* group significantly ($P = 0.000$) higher at 0.73 ± 0.03 mmol/l than that for LSD at 0.42 ± 0.02 mmol/l. Interestingly, plasma calcium mean ± SD ion concentration for HSD group at 0.13 ± 0.00 mmol/l was significantly (P = NaN) not different from that of LSD group at 0.13 ± 0.00 mmol/l. Na+ and K+ ions concentrations showed significant variations in the means ± SD between both control and experimental *O. niloticus* groupings, there was zero difference in the same means for Ca^{++} ion concentration.

The elevated Na^+ concentration, 1.63 ± 0.18 mmol/l realized in this study is consistent with significant increase in Na^+ ion concentration levels recorded from *O. nilotiocus* directly transferred out of freshwater in to 18 ppt salinity (Karsi and Yildiz, 2005). The higher plasmic sodium ion concentration recorded in HSD group contradicts the biological principle that, the adjustment in the permeability of the gill as a result of the effect of catecholamines produced due to stress (Barton and Iwama, 1991) should have plasmic sodium shift to water (Biller *et al*., 2008) thus decreasing the amount of the ion in HSD group to below that of LSD *O. niloticus* group.

Wendelaar-Bonga, (1997) reports that numerous stressors impact the hydro-mineral balance in fish by enhancing the gill epithelial`s permeability to water and ions which triggers a systematic hydromineral levels disturbances. In freshwater fishes, the osmotic water influx and diffusive losses of ions, such as Na^+, Cl^- and Ca^{++}, are compensated by the excretion of large volumes of dilute urine and the active uptake to replace lost ions by the branchial chloride cells. A disturbance of mineral balance occurs in juvenile *Piaractus mesopotamicus* experiencing air exposure induced stress (Biller *et al*., 2002). In some freshwater fish species, handling and capture procedures trigger physiologic decrease in plasma sodium ions (Carneiro and Urbinati, 2001) and increased concentrations of plasma potassium (Carneiro and Urbinati, 2002; Biller *et al*., 2002) showing sensitivity of these ions to stressors.

It should be noted that, since the elevated Na^+ ion concentration in the HSD *O. niloticus* groups, contradicts the biological principle on the influence of catecholamines on the gill permeability of stressed fish, with sodium pump moving ions out of the gills into the water (Biller *et al*., 2008). Furthermore, the elevated Na^+ ions concentration in this study is not consistent with the findings reported for freshwater fish; *Oncorhynchus kisutch* (Redding and Schreck, 1983), *Brycon cephalus*

(Carneiro and Urbinati, 2002**),** *Oreochromis niloticus*(Karsi and Yildiz, 2005) thus, it cannot be reliably deployed as a stress indicator in this study.

Several authors have reported increased levels K^+ ions concentration in stressed fish which is in agreement with outcome of this study. Carneiro and Urbinati (2001; 2002) reports increased serum potassium levels after transport of *Brycon cephalus*. A significant increase in K^+ ions occurred in seawater adapted tilapia, *Seratherodon melanotheron* and in *Oreochromis niloticus* after transferring them directly to waters of varied salinities (Karsi and Yildiz, 2005). In a study, Martemyanov (2014) observed a 76 % increase in the levels K^+ in plasma after two hours exposure of freshwater species roach and bream to the stress, The plasma also exhibited coloration probably caused by red blood cells haemolysed due to stress in *O. niloticus,* buttressing the theory that the plasma lemma ruptures in fish experiencing stress (Biller *et al*., 2008). Thus, the surge in plasma potassium ion concentration levels reported in this study could linked to increased fragility of the red blood cells provoked by stress, which could have brought about the hemolysis of the cells during the processing of blood. This presents potassium ion concentration as a biomarker of stress in fish aquaculture.

Although Ca^{++} is not a major electrolyte in blood plasma (2 mmol L^{-1}) relative to Na^+ (150 mmol L^{-1}) in freshwater fishes, this ion is essential in many physiological processes, such as control of membrane permeability, activation of muscular contraction, transmission of nervous impulses and bone formation. Biller *et al*., (2008) observes that, calcium has not been usually interpreted as a stress indicator. This supports the findings of this study since stress was found to completely have no influence on the levels of plasma Ca^{++} ion concentration, however the levels Ca^{++}has been usedas a test for the functions of the liver and the metabolism of calcium (Zhou *et al.*,2009).

5.4 The Influence of the Study Limitations on the Results

Three limitations whose impact on the study results are discussed below were identified.

5.4.1 Handling Induced Stress During Sampling

The fish plasma biochemistry was impacted on by pre-sampling practices which could have biased the findings realized from this study if they were disregarded within the general sampling strategy with the possibility of acute handling stresses being introduced in fish during capture. For this study, the fish were sedated using 2 – phenoxyethanol 10 minues before sampling. Young *et al.*, (2019) observes that, fish are commonly immobilized through sedation and/or euthanisation to alleviate stress, pain or suffering prior to anon – lethal blood sample extraction. They corroborated this through a multivariate analyses of biochemical targets comprising ions, metabolites, and enzymes in plasma of Chinook salmon, *Oncorhynchus tshawytscha* that showed two minutes of confinement with mild handling manipulation returning a significant shift from metabolic baseline with more exasperation during an extended five-minute provocation/challenge. These adjustments were found to disrupt osmoregulation, switch towards oxygen-independent metabolism, and shifts in the recycling of ammonia, among others. In this study, results demonstrate consistency and reliability across the hematological and biochemical parameters sincere-harvest practices were carefully managed and standardized during the sampling of fish for hematological/biochemical-based evaluations with manipulations unavoidable.

5.4.2Precision Error in the Erythrocyte Count

In this study, the precision error in the erythrocyte count was significantly reduced by counting all the border cells and then dividing by two. This improved counting method reduced the counting

error of the Neubauer counting chamber significantly assessing the distribution uniformity of border cells, and thus eliminated the samples with large differences in distribution.

5.4.3 The Scope of Hematological; Erythrocyte Count and Biochemical, Plasma Cortisol, Whole Blood Glucose, and Electrolytes Parameters

This study investigated only four parameters as biomarkers of stocking density induced stress in *O. niloticus*. This could limit the space on the options on biochemical markers available in the management of stress by tilapia farmers since the study did not consider a number of other haematological, biochemical and cellular Heat shock proteins as markers of stress in fish and thus furthers studies should investigate these parameters.

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1. Summary

The study showed that there were changes in the evaluated hematological and biochemical indices in *O. niloticus* blood which may cause biochemical dysfunction in this species. In addition, results provided evidence that hematological and biochemical parameters can be sensitive to stocking density induced stress thus can be used as indicators of stress in fish. The outcomes of this study demonstrate the fact that HSDs have significant impact on erythrocyte counts, plasma cortisol, whole blood glucose and blood electrolytes concentrations in tilapia fish. The elevated erythrocyte count, plasma cortisol, whole blood glucose and electrolyte concentrations except Ca^{++} ion concentration indicated that chronic stress induced by high stocking density occurred in the experimental fish. Elevated erythrocyte count could have been a response to increase the oxygen loading capacity to various tissues. In addition, it can be further argued that, the fish suffered increased glycogenolysis or a decreased clearance of glucose from the blood thus raising plasma glucose concentration in the tilapia mediated by cortisol. It was evident that cortisol concentration in plasma elevated in response to stress and aided in carbohydrate metabolism and promoted glucogenesis. The findings of this study can help inform policy on the management of stress caused by overpopulation of *O. niloticus* and other related Cichlids under industrial aquaculture production.

6.2. Conclusions and Implications

It's evident that HSD had a significant effect on the elevation of Plasma cortisol, whole blood, electrolyte levels and the erythrocyte counts. The mean concentration values of these parameters for HSD were found to be significantly higher than in LSD save for Ca++ which did not register any deviations in the mean concentration for the two groups of *O. niloticus*. Since these parameters were elevated in HSD *O. niloticus* groups, it's therefore correct to associate the increase in their concentrations to high stocking density induced stress experienced by the fish. These parameters can therefore be used to help inform the policy interventions for the management of stress in *O. niloticus* and other related Cichlids under industrial aquaculture production.

6.3. Recommendations

6.3.1. Recommendations from the present study

The study revealed elevations in the concentrations levels of all the studied hematological and biochemical parameters but Ca^{++} ions in the experimental fish. It is therefore evident that these parameters; Erythrocyte count, Plasma Cortisol, Whole Blood Glucose, Sodium and Potassium ion but calcium ion concentrations can therefore be used as biomarkers of chronic stress in *O. niloticus* production under aquaculture systems in the study area.

6.3.2: Recommendations for Future Studies

It's recommended as follows for future studies;

1) That chronic stress elicits elevated levels of all the studied hematological and biochemical parameters but Ca^{++} ions in the experimental fish and thus it will be important to:

a) Conduct a study on how the physiological mechanisms would account for the low corticosteroid stress responses under similar experimental conditions
b) Conduct a study to establish why the concentration of Ca^{++} ions remained statistically the same under both low and high stocking densities, and if stress has any meditative mechanisms on Ca^{++} elevations in *O. niloticus* subjected to stocking density induced stress.
c) Conduct a study to evaluate for a base line for erythrocyte concentration in Nile tilapia for aquaculture systems in the study area since a few studies have given contrary results, apparently because these values may depend on a number of extrinsic factors i.e., water characteristics such as salinity, hydro-mineral balance, temperature etc.

2). That more haematological indices, such as Haemoglobin (Hb), Packed cell volume (PCV), White blood cell (WBC), Mean corpuscular volume (MCV) and Mean corpuscular hemoglobin (MCH) should be infused into a similar future study for evaluation of fish health conditions.

3). That cellular alternate biomarkers of stress could also be studied on Nile tilapia in the study area to buttress the studied biomarkers.

4). A similar study should be conducted on Nile tilapia with established genetic pools i.e., pure strains, improved strains amongst other groups in the study area

5). A similar study should be conducted on Nile tilapia with a larger sample size.

REFERENCES

Abdel-Tawab M., (2012). Effects of dietary protein levels and rearing density on growth Performance and Stress Response of Nile tilapia. *Oreochromis niloticus* (L.). *Int Aqua Res*.**4**, 3.

Abdel – Tawwab M., Mousa M. A. A., Sharaf S. M and Ahmad M. H. (2005). Effects of crowding stress on some physiologial functions of Nile tilapia, *Oreochromis niloticus* (L) Fed on different dietary protein levels. *Intern J Zool.Res.***1** (1), 41-47.

Abidin A. S. Z, Rahim R. A., Arshad M. K. M.,Nabilah M, F. F., Voon C. H., Thean-Hock T and Citartan M., (2017). Current and potential developments of cortisol aptasensing towards Point - of - CareDiagnostics (POTC). *MDPI*. **17** (5), 1180

Abou Y., Flogbe E and Micha J., (2007). Effects of stocking density on growth, yield and profitability of farming Nile tilapia, *Oreochromis niloticus* L., fed *Azolla* diet, in earthen ponds. *Aquac Res*. **38**, 595-604.

Abreu J.S., Takahashi L.S., Hoshiba M. A and Urbaniti, E.C., (2009). Biological indicators of stress in Pacu (Piaractus *mesopotamicus*) after capture. *Braz J Biol*.**69** (20), 415 – 421.

Acosta B. O and Gupta M. V., (2009). The Genetic improvement of farmed Tilapias project: impact and lessons learned. *Success Stories in Asian Aquaculture*. http://www.idrc.ca/en/ev-147123-201-1-DO_TOPIC.htm

Ackerman P. A., Forsyth R. B., Mazur C. F. and Iwama G. K., (2000). Stress hormones and the cellular stress response in salmonids. *Fish Physiol. Biochem.* **23**, 327-336.

Akhtar M. S., Farooq A.A and Mushtaq M., (2009). Serum trace minerals validation during pre and post-partum period in Nili-ravi Buffuloes. *J Anim & Plant Sci*. **19**, 182-184.

Aketch B. O., Ang`ienda P. O., Radull J. O and Waindi E. N., (2014). Effect of stocking density on the expression of glucose tramsporter protein 1 and other physiological factors in the Lake Victoria Nile tilapia, *Oreochromis niloticus* (L.).*Int. Aquat. Res*. **6**, 69.

Aluru N and Vijayan M. M., (2009). Stress transcriptomics in fish. A role for genomic cortisol signaling. *Gen. Comp. Endocrinol*.**164**, 142-150.

Arends R. J., Mancera J. M., Muňoz J. L., Wendelaar-Bonga S. E., and Flik G., (1999). The stress response of the of gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. *J Endocrinol*. **163**, 149-157.

Avella, M., Berhaut J and Bornancin M.,(1993). Salinity tolerance of two tropical fishes, *Oreochromis aureus* and *Oreochromis niloticus*. I. Biochemical and morphological changes in the gill epithelium. *Jl of Fish Biol*. **42**,243-254.

Balasch J. C and Tort L., (2019). Netting the stress responses in fish. *Front. Endocrinol.***10**, 62.

Balci A. K., Koksal O., Kose A., Amargan E., Ozdemir F., Inal T and Oner N., (2013). Geeral Characteristics of Patients with Electrolyte Imbalance admitted to Emergency Department. *World J Emerg Med*. **4**(2); 113-116.

Barcellos L.J.G., Nicolaiewsky S., De Souza S.M.G and Luther F., (2001). The effects of stocking Density and Social Interaction on Acute Stress Response in Nile tilapia, *Oreochrom*is *niloticus* (L) fingerlings. *J Aquac Res*.**30**, 887-892.

Barreto R. E and Volpato G. L., (2006). Stress responses of fish Nile tilapia subjected to electroshock and social stressors. *Braz J Med Bio Res*. **39**(12), 1605-1612.

Barton B. A., (2002). Stress in Fishes: A diversity of responses with particular reference to change in circulating corticosteroids. *Integ CompBiol*. **42**, 517-525.

Barton B. A and Iwama, G.K., (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Anim Rev. Fish Dis*.**10**, 3-26.

Becker A. G., Goncalves J. M., Toledo J. dos A., Burns M. D. M., Garcia L. de O., Veiras J. P and Baldisserotto B., (2011). Plasma ion levels of freshwater and marine/estuarine teleosts from Southern Brazil. *Neotropical Ichthyology*. **9**(4), 895-900.

Bentov Y and Werner H., (2008). IGF-1R (insulin-like growth factor 1 receptor). *Atlas Genet Cytogenet Oncol Haematol.* pp 2.

Benttencourt N. de L. R., Molinari L. M., Oliver de D., Pedro R. B., Nakamura C. V., Ueda-Nakamura T., Filho B. A. de A and Filho B. P. D., (2003). Haematological and biochemical values for Nile tilapia *Oreochromis niloticus* cultured in semi-intensive system.*Acta Scientiarum. Biological Sciences. Maringa*, **25**, 2, 385-389.

Biller J. D., Bendhack F., Takahashi L. S and Urbinati E. C., (2008). Stress responses in Juvenile pacu (*Piaractus mesopotamicus*) submitted to repeated air exposure. *Acta Sci. Anim. Sci*.**30**, 89-93.

Biswas A. K., Maita M., Yoshizaki G and Takeuchi T., (2004). Physiological responses in Nile tilapia exposed to different photoperiod regimes.*J Fish Biol*. 65, 811-821.

Bockenkamp B and Vyas H., (2003) Understanding and managing acute fluid and electrolyte disturbances. Current paediatrics. *Science Direct, Elsevier*. **13**:520–528

Braun T. P and Marks D. L., (2015). The regulation of muscle mass by endogenous glucocorticoids. *Front. Physiol.***6**, 12.

Campbell T. W (1995). Avian hematology and cytology. Second Edition. *Iowa State University press.*

Carneiro P. C. F and Urbinati E. C., (2001). Electrolyte disturbance in Matrinxã *Brycon cephalus* following transport stress under benzocaine effect. *J. Appl. Aquacult*., Binghamton. 11 (4), 1-13.

Carneiro P. C. F and Urbinati E. C., (2002). Ionic imbalance in Matrinxã,*Brycon cephalus* (Teleostei: Characidae), Submitted to different hauling densities. *Aquacult. Intern*. London. 10 (3), 221-229.

Charo-Karisa H, Rezk, M. A, Bovenhuis, H and Komen, H., (2005). Heritability of cold tolerance in Nile tilapia, *Oreochromis niloticus*, juveniles. *J. Aquac*. **249**, 115-123.

Chen C., Wooster G. A and Bowser P. R., (200). Comparative blood chemistry and histopathology of tilapia infected with *Vibrio vulnificus* or *Streptococcus iniae* or exposed to carbon tetrachloride, gentamicin or copper sulfate. *Aquaculture.***239**, 421-443.

Chrousos G. P., (2009). Stress and disorders of the stress system. *Nat Rev Endocrinol*. **5**(7):374-81.

Chrousos G.P and Gold P.W., (1992). The concept of stress and stress system disorders. Overview of physical and behavioural homeostasis. *J Am Assoc.***267**, 1244-1252.

Cnaani A. S., Avidar Y., Ron M and Hulata G., (2004). Comparative study of biological parameters in response to stress *in Oreochromis aereus, Oeochromis mossambicus* and two strains of *O. niloticus*. *J. AquacRes*. **35**, 1434-1440.

Conte F. S., (2004). Stress and the welfare of cultured fish. *Appl. Anim. Behav. Sci*. **88**, 2005-223.

Crain D. A., Gross S. T., Cox M.C and Guillette L. J. Jr, (1995). Insulin-like growth factor-1 in the plasma of two Reptiles: Assay development and validations. *Gen Comp Endocrinol***. 98**, 26-34.

Crane D. P., Ogle D. H. and Shoup D. E., (2019). Use and misuse of a common growth metric; guidance for[Ma1] appropriately calculating and reporting specific growth rate.*Reviews in Aquaculture*, 1-6.

De Pedro N., Guijarro A. I., Lopez-Patino M. A., Martinez-Alvarez R., and Delgado M. J., (2005). Seasonal variations in hematological and blood biochemical parameters in the tench, *Tinca tinnca* Linnaeus 1758. *Aquqcul. Res*, **36** (12), 1185-1196.

Duan C., (1997). The Insulin-Like Growth Factor System and its biological actions in Fish. *Amer Zool*. **37**, 394-503.

Dykstra M.J and Kane A.S., (2000). *Pfiesteria piscicida* and ulcerative myosis of Atlantic Menhaden- current status of understanding. *J Aquat Anim Health.***12**, 18-25.

Duan C and Xu Q., (2005). Role of insulin‐like growth factor (IGF) binding proteins in regulating IGF actions. *Gen Comp Endocrinol*. **142**, 44-52.

Elarabary N., Bahnasawy M., Edrees G and Alkazagli R., (2017). Effects of salinity on some hematological and biochemical parameters in Nile tilapia, *Oreochromis niloticus*. *Agric, Forest. and Fish*. **6**(6), 200-2005.

Electron Microscopy Sciences (2020). Technical data sheets. Neubauer heamocytometry. EMS Catalog# 68052-14, 68052-15.

Elies G., Duval H., Bonnec G., Wolff J, Boeff G and Boujard D., (1999). Insulin and Insulin-Like Growth Factor-1 receptors in an evoluted fish, the turbot: cDNA cloning and mRNA expression. *Mol Cellul Endocrin*. **158**, 173-175.

El–Sayed A., (2002). Effects of stocking density and feeding levels on growth and feed efficiency of Nile tilapia (*Oreochromis niloticus* L.) fry. *Aquac Res.* **33**, 621-626.

El–Zarka S., Shaheen A.K and Aleem A. A., (2007). Tilapia fishery in Lake Mariut; age and growth of Tilapia niloticus in the lake. http://el.erdc.usace.army. Mill/ansrp/ANSIS/html orechromis_niloticus_nile_tilapia.htm.

Evans J. J., Pasnik D. J., Horley P. J., Kraeer, K. J and Klesius P. H., (2008). Aggression and mortality among Nile tilapia (*Oreochromis niloticus*) maintained in the laboratory at different densities. *Res J of Anim Sci*.**2** (2), 57-64.

Evans J. J., Klesius P. H., Shoemaker C. A and Fitzpatrick B. T., (2004). *Streptococcus agalactiae* vaccination and infection stress in Nile tilapia, *Oreochromis niloticus*. *J. App. Aqua.***16,** 105-115.

FAO, (2009). Fisheries and Aquaculture *Oreochromis niloticus* www.org/fishery/culturedspecies/oreochromis_niliticus/en

FAO, (2010). Fisheries and Aquaculture. *Oreochromis niloticus.* http://www.fao.org/fishery/culturedspecies/oreochromis_niloticus/en

Francis-Floyd R., (2009). Stress-Its Role in Fish Disease. *The Institute of Food and Agricultural Sciences (IFAS).* University of Florida. Gainesville, 32611.

Getabu A., (1992). Growth parameters and total mortality in *Oreochromis niloticus* (Linnaeus) from Nyanza gulf, Lake Victoria. *Hydrobiologia***. 232**, 91-97.

Getinet G. T., (2008). Effects of maternal age on fecundity, spawning interval, and Egg quality of Nile tilapia, *Oreochromis niloticus* (L.). *J World AquacSocie*.**39** (5), 671-672.

Gomes L. de C, Roubach R, Cavero B.A.S, Pereira-Filho M and Urbinati E.C., (2003). Transport of Pirarucu, *Arapaima gigas* Juveniles in plastic bag. *ACTA AMAZONICA*.**33** (4), 637 – 642.

Goncalves-de-Freitas E and Mariguela T. C., (2006). Social Isolation and Aggressiveness in the Amazonian Juvenile Fish. *Astronotus ocellatus*. *Braz. J. Biol*.**66** (1B), 233-238.

Goncalves-de-Freitas E and Nishinda, S.M., (1998). Sneaking Behavior of the Nile tilapia. *B. Tec. CEPTA, Pirassununga*. 71- 79.

Greenwell M. G., Sherill J and Clayton L. A., (2003). Osmoregulation in fish mechanisms and clinical implications. *Vet Clin Exot Anim*. 6, 169–189.

Hassanien H. A and Gilbey J., (2005). Genetic diversity and differentiation of Nile tilapia (*Oreochromis niloticus*) revealed by Microsatellites. *J. Agric Res* .**36**, 1450-1457.

Inoue L.A.K.A, Morales G. Iwama G.K and Afonso L, O, B, (2008). Physiological stress responses in the warm – water fish Matrinxa (*Brycon amazonicus*) subjected to a sudden cold shock. *ACTA AMAZONICA*. **38** (4), 603 – 610).

Iwama G.K., (2002). Stress in fish. A tribute in Dave Randall, Mentor and Friend. *Institute for Marine Biosciences, Natural Research Council of Canada*, 141 of Oxford Street, Halifax, Nova Scotia.B3H 321.

Iwama G.K., Afonso L., Todgham A., Paige A and Nakano K., (2004). Are Heat shock proteins reliable indicators of general stress in fish? *J Exp Biol.***207**, 15-19.

Iwama G.K., Afonso L. O. B and Vijayan M. M., (2004). AquaNet workshop on fish welfare, Campbell River, B. C. Canada.

Iwama G.K., Vijayan M. M., Forsyth R.B and Ackerrman P.A., (1999). Heat shock protein and physiological stress in fish. *Amer. Zool.***39**, 901 – 909.

Jimmy W., (2010). *Oreochromis niloticus*. Wikipedia the free encyclopedia. http://en.

wikipedia.org/wiki/ *Oreochromis_niloticus*.

Kalamarz-kubiak H., (2017). Cortisol in correlation to other indicators of fish welfare. DOI: 10.5772/intechopen.72392.

Kamal, S.M and Omar, W.A. (2011). Effect of different stocking densities on hematological and biochemical parameters of Silver Carp, *Hypophthalmichthys molitrix* fingerlings. *Life Science J.* **8,** 580-586.

Karsi A and Yildiz H. Y., (2005). Secondary stress response of Nile tilapia (*Oreochromis niloticus*), after direct transfer to different salinities. *Tarim Bilmleri Dergisi.***11** (2), 139-141.

Kefas M., Abubakar K. A and Ja`afaru A., (2015). Haematological indices of tilapia (*Oreochromis niloticus*) from Lake Geriyo, Yola, Adamawa State, Nigeria. *Inter. J. Fish. Aqua. Studies*. **3**(1), 09-14.

KEMFRI (2018). Bounties of Lake Victoria. Cage culture real story. Make money through cage fish farming in Lake Victoria. *Kisumu CenterNewsletter*.**1** (1).

Khan E. A., Dasmahapatra A. K. and Ghosh R., (1997). Evaluation of EDTA and fish skin extract in primary culture of fish liver cells. *J Methods in Cell Sci*. **19** (3), 153-159

KNBS (2019). Kenya population and housing census results. *Kenya National Bureau of Statistics.*

Kocher T. D., Soboleswa H. Penman D and McAndrew B., (1998). A Genetic linkage map of a cichlid fish, the Nile tilapia (*Oreochromis niloticus*). *J. Genetics*, 148, 1225-1232.

Koeypuda W and Jongjareanjai M., (2011). Impact of water temperature and sodium chloride (NaCL) on stress indicators of hybrid catfish (*Clarias gariepinus* Burchell x *C. macrocephalus* Gunther). *Songlanakarin J. Sci. and Tech*. **33** (4), 369-378.

Kubokawa K. Watanabe T. Yoshioka M. and Iwata M., (1999). Effects of acute stress on plasma cortisol, sex steroid hormone and glucose levels in male and female Sockeye Salmon during the breeding season. *Aquac.* 172, 335-349.

Lebendiker M., (2002). PAGE-SDS Laemmli Protocol. The protein purification facility. The Wolfson Centre for Applied Structural Biology. The Hebrew University of Jerusalem. mariol@mail.ls.huji.ac.il.

LeRoith D., Bondy C., Yakar S., Liu J. L and Butler A., (2001). The somatomedin hypothesis. *Endocr Rev*. 22, 53-74.

Li S. F., Zhao J. L., Dey M. and Dunham R., (2001). Isozyme variation of Nile tilapia (*Oreochromis niloticus*) in China.*J. Asian Fish Sci*. 14, 411-416.

Lipson L. G., Capuzzi D. M. and Margolis S., (1972). Effect of method of cell isolation on the metabolic activity of isolated rat liver cells. *J. Cell Sci*. **10**, 167-179.

Luomba, J. O (2007) Monitoring of socio economic status report. *IFMP Technical report.*

Luomba J., Mwanahamisi S. and Mlahagwa (2013). An assessment of the socio-economic status of fishers` communities in Lake Victoria, Tanzania in relation to poverty. *Int. J of Reserch in Fish. and Agric*. **3**, 98-102.

MacDonal O., Chediack J. G. and Caviedes-Vidal E., (2008). Isolation of epithelial cells, villi and crypts from small intestine of Pigeons (*Columba livia*). *Biocell*. **32** (3), 219-227.

Magnoni L. J.,Novais. S C., Eding E., Leguen I., Lemos F. M. L., Ozorio R. O. A., Geurden I., Prunet P. and Schrama J, W., (2019).Acute stress and an electrolyte – imbalanced diet, but not chronic hypoxia, increase oxidative stress and hamper innate immune status in Rainbow trout(*Onchorhynchus mykiss*) isogenic line. *Front. Physio*.**10**, 453.

Mohanty B. P., Mahanty A., Mitra T and Parija S., (2018). Heat shock proteins in stress in Teleosts. *ResearchGate*. **13**.

Martemyanov V, I., (2014). Dynamics of sodium and potassium in plasma, erythrocytes and muscles of freshwater species under the effect of long-term combined stress. *Ecol. Physoil. Biochem. Hydrobiontics.***7** (4), pp 78-82.

Martemyanov V. I., (2013). Patterns of changes in sodium content in plasma and erythrocytes of freshwater fish at stress. *J. Ichthyology*. **53**, 220-224.

Martinez–Porchas M and Martinez–Cordova L. R., and Ramos–Enriquez R., (2009). Cortisol and glucose: reliable indicators of fish stress? *Pan–American J Aquat Sci*. **4**(4), 158-178.

Martins M. L., Mouriño J. P., Amaral G. V., Vieira F. N., Dotta G., Jatobá A. M. B., Pedrotti F. S., Jerônimo G. T., Buglione-Neto C. C and Pereira-Jr. G., (2008). Haematological changes in Nile tilapia experimentally injected with *Enterococcus* sp. *Braz. J. Biol*. **68**(3), 657-661.

Masterson J., (2007). Mosambique tilapia, *Oreochromis mossambicus*. *Smithsonian Marine Station at Fort Pierce*. irl_webmaster@si.edu.

Mjoun K., Rosentrater K and Brown M. L., (2010). Tilapia: Environmental biology and nutritional requirements. *SDSU Extension*.Access athttp://pubstorage.sdstate.edu/AgBio_Publications/articles/FS963-02.pdf**.**

Mommsen T. P., Vijayan M. M and Moon T. W., (1999). Cortisol in teleost, dynamics, mechanisms of action, and metabolic regulation. *Rev Fish Biol Fisheries*. **9**, 211-268.

Morales A. E., Cardenete G., Abellan E and Garcia-Rejon L., (2005). Stress-related physiological responses to handling in cmmon dentex (*Dentex dentex* Linnaeus, 1758). *Aquaculture Research*. **36**. 33-40.

Moreira P. S. A and Volpato G. L., (2004). Conditioning of stress in Nile tilapia. *J Fish Biol*. **64**, 961-969.

Munguti J., Obiero K., Orina P., Mirera D., Kyule D., Mwalumu J., Opiyo M., Musa S., Ociewo J., Njiru J., Ogello E and Hagiwara A., (2021). State of aquaculture report 2021; towards nutrition sensitive fish food production systems. *Techplus Media House*, Nairobi, Kenya, pp 190.

Nandlal S. and Pickering T., (2004).Tilapia fish farming in Pacific Island Countries.Tilapia hacheries operation.Noumea New Caledonia: *Secretariat of the Pacific Community.* Vol. **1.**

Ndong D., Chen Y., Lin Y., Vaseeharan B and Chen J., (2007). The immune response of tilapia *Oreochromis mossambicus* and its susceptibility to *Streptococcus iniae* under stress in low and high temperature. *Fish & Shellfish Immunology*. **22**. 686-694.

Neogen Corporation, (2010). Cortisol ELISA Kit Instructions. Product # 402710, USA.

Nirupama R., Rajaraman B and Yajurvedi H. N., (2018). Stress and glucose metabolism: A Review. *Imaging J Clin. Medical Sci*. **5**(1), 008-012.

Njiru M., Ojuok J., E, Okeyo-Owuor J. B., Muchiri M., Ntiba M. J. and Cowx I. G., (2006). Some biological aspects and life history strategies of Nile tilapia, *Oreochromis niloticus* (L) in Lake Victoria, East African Wildlife Society. *Afr. J. Ecol.***44**, 30-77.

Odhiambo E., Angienda P. O., Okoth P and Onyango D., (2020). Stocking density inducedstress on plasma cortisol and whole blood glucose concentration in Nile tilapia fish (*Oreochromis niloticus*) of Lake Victoria, Kenya. *Int. J Zoology*. vol, **2020**.

Oke V and Goose N. J., (2019). Feeding effect on viscera of marine fish and broiler on the production of African Cat fish Clarias grepinus (Burchell, 1822). **507.**,

Old J. M. and Huveneers C., (2006). Morphology of the blood cells from three species of wobbegong sharks (*Orectolobus* species) on the east coast of New South Wales. *Zoo. Biol*. **25**, 73-82.

Ombwa V., Aura C., Odada E., Ogari Z., Ogik W., Ogwai K., Chelangat M., Achieng M and Onienga P., (2018). The Socio-economic IMPACT OF cage culture in Lake Victoria for informed decision making. Technical report; KMF/RS/2017/C1.8 (ii).*Kenya Marine and Fisheris Research Institute*, pp 57.

Onyango, P. O., Salehe, M and Mrosso, H., D., J., (2006). Socio economic baseline survey report. *IFMP Technical report*

Ortuno J., Esteban M. A and Mesengner J., (2001).Effects of short-term crowding stress on gilthead sea bream (*Sparus aurata* L.) innate immune response. *Fish Shellfish Immunol.***11**, 187-197.

Osofero S., Otubusin S and Daramola J., (2009). Effect of stocking density on tilapia (*Oreochromis niloticus* Linnaeus 1757) growth and survival in bamboo. *Afric. J. Biotech*. **8** (7) 1322-1325.

Osman A., G., M., AbouelFadl, K., Y., Baset A., E., M., Reheem A., E., Mahmoud U., M., Kloas W and Moustafa M., A., (2018). Blood biomarkers in Nile tilapia *Oreochromis niloticus* and African Catfish *Clarias gariepinus* to evaluate water quality of the River Nile. *J. of Fish. Sci.***12** (1), 001-015.

Pangni K., Atse B.C and Kovassi N. J., (2008). Effect of Stocking Density on Growth and Survival of the African catfish *Chrysichthys nigrodigitus*, Claroteidae (Lacepede 1803) larvae in circular tanks. *Livestock Research for Rural Development.***20** (7).

Parrizas M., Plisetskaya M. E., Planas J and Gutierrez J., (1995). Abundant Insulin –Like Growth Factor – 1 (IGF–1) Receptor Binding in Fish skeletal muscle. *Gen Comp Endocrinol*. **98**, 16-25.

Pasnik D. J., Evans J. J and Klesius P. H., (2008). Influnce of Tricane methanesulfonate on *Streptococcus agalactiae* Vaccination of Nile tilapia *(Oreochromis niloticus*). *Vet. Res.***2** (2), 28-33.

Perry L and Medbak S., (2013). The adrenal cortex. *The Immunoassay Handbook. Science Director*. Fourth Edition.

Perrot V., Moiseeva E. B., Gozes Y., Chan S. J., Ingleton P. S and Funkenstein B., (1999). Ontogeny of Insulin–like Growth Factor System (IGF–1, IGF–11, and IGF–1R) in Gilthead Seabream (*Sparus aurata*): Expression and cellular localization. *Gen Comp Endocrinol.***116**, 445-460.

Peter M. S & Simi, S., (2017). Hypoxia stress modifies Na^+/K^+-ATPase, H^+/K^+-ATPase, [Formula: see text], and *nkaα1* Isoform expression in the brain of immune-challenged air-breathing fish. *Journal of experimental neuroscience*, **11**, 1179069517733732. https://doi.org/10.1177/1179069517733732.

Pillay T. V. R., (1980). Aquaculture development and coordination programme. Fish technologist of ADP. Rome.

Pickering A. D., (1993). Growth and stress in fish production. *Aquaculture.***111**, 51-63.

Popma T and Masser M., (1999). Tilapia, life history and biology. *SRAC* No. 283.

Portz D. E., Woodley C. M and Cech J. J., (2006). Stress-associated impacts of short-associated impacts of short-term holding on fishes. *Rev. Fish Biol. Fish*. **16**, 125-170.

Ramamoorthy S and Cidlowski J. A., (2016). Corticosteroids: mechanisms of action in Health and Disease. *Rheum. Dis. Clin. North Am*. **42**(1), 15-31

Rebl A., Zebunke M., Borchel A., Bochert R., Verleih M and Goldammer T., (2017). Microarray-predicated marker genes and molecular pathways indicating crowding stress in Rainbow trout (*Onchorhynchus mykiss*). *Aquaculture.***473**, 355-365.

Redding J. M and Schreck C. B., (1983). Influence of ambient salinity on osmoregulation and cortisol concentration in yearling Coho salmon during stress. *Trans. Am. Fish. Soc*.,**112**, 800-807.

Reid S. G., Bernier N. J and Perry, S. F., (1998). The adrenergic stress response in fish. Control of catecholamine storage and release. *Comparative Biochemistry and Physiology Part C.* **120,** 1-27

Ridha M. T., (2006). Comparative study of growth performance of three strains of Nile tilapia, *Oreochromis niloticus*, L. at two stocking densities. *Aquac Res*. **37**, 172-179.

Rojas A and Wadsworth S., (2007). A review of cage aquaculture; Latin America and the Caribbean. *Cage Aquac*. – Reg. Rev. Glob. Overv. pp 77-100.

Romano G., (2003). The complex Journal of Prous science Biology of the Receptor for the Insulin-Like Growth Factor-1. *News Drug Perspect*. **16**. (8), 525.

Ronald N., Gladys B and Gasper E., (2014). The effects of stocking density on the growth and survival of Nile Tilapia (*Oreochromis niloticus*) fry at Son fish farm, Uganda. *J. Aquaculture*. **5**, 2.

Rotllant J and Tort L., (1997). Cortisol and glucose responses after acute stress by net handling in the sparid red porgy previously subjected to crowding stress. *J Fish Biol*.**51,** 21-28.

Schmid A. C., Näf E., Kloas W and Reinecke M., (1999). Insulin–like growth factor–1 and -11 in the ovary of a bony fish, *Oreochromis mossambicus*, the tilapia: in situ hybridization, immunohistochemistry localization, Northern blot and cDNA squences. *MolCellul Endocrin.***156**, 141-149.

Schreck B. C., Contreras–Sanchez W and Fitzpatrick M. S., (2001). Effects of stress on fish reproduction, gamete quality, and progeny. *Aquaculture.***197**, 3-24.

Sebastiáo F. A., Nomura D., Sakabe R and Pilarski F., (2011). Hematology and productive performance of Nile tilapia (*Oreochromis niloticus*) naturally infected with *Flavobacterium columnare*. *Braz. J. Biol*. **42**, 282-289.

Seriani, R., Abessa, D.M.S., Kirschbaum, A.A., Pereira, C.D.S and Romano, P., (2011). Relationship between water toxicity and hematological changes on *Oreochromis niloticus*. *Brazilian J Aquatic Sci Technol*. **15,** 47-53.

Shen Y., Wang D., Zhao J and Chen X., (2018). Fish red blood cells express immune genes and responses. *Aquaculture Fisheries*. **3**, 14 – 21.

Sherwood Scientific LTD (2004). Model 410 and 410 C. Flame Photometer Operation manual. Issue **6**.

Smith S. M and Vale W. W., (2006). The role of the hypothalamic-pituitary adrenal axis in neuroendocrine responses to stress. *Dialogues in Clinical neuroscience*. **8**(4), 383-395.

Stouthart A. J. H. X., Lucassen E. C. H. E. T., Van-Strien F. J. C., Balm P. H. M., Lock R. A. C and Wandelaa-bonga S. E., (1998). Stress responsiveness of the pituitary-interrenal axis during early life stages of Common Carp (*Cyprinuscarpio*). *J Endocrnol*. **157**, 127 – 137.

Tavares-Dias M., Sandrim E., F., da S., Morales F., R and Carneiro P., C., F., (2001). Physiological response "Tambaqui"*Colossoma macropomum* (Characidae) To Acute Stress. *Boletim do Instuto de Pesca. São Paulo.***27** (1), 43-48.

Than L., Gandhi J and Sharma S., (2020). Physiology, cortisol. StartPearls. *Publishing LLC.*

Thau L., Gandhi J and Sharma S., (2020). Physiology, cortisol. *StatPearl* (Internet).

Tsigos C., Kyrou L., Kassi E. M. D and Chrousos G. P., (2016). Stress, endocrine, physiology and pathology. *Endotex.* South Dartmouth (MA): MDText.com, Inc.; 2000-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK278995

Umbrello M., Dyson A., Feelisch M and Singer M., (2013). Antioxidants &redox signaling. *Mary Ann Liebert, inc. Publishers*. **19** (14), 1690-1710.

Veĺišek J., Svobodova Z and Piaĉkova V., (2007). Effects of 2-phenoxyethanol anaesthesia on haematological profile on Common Carp (*Cyprinus carpio*) and Rainbow Trout (*Oncorhynchus mykiss*). *Acta Vet. Brno*. **76**, 487-492.

Viana P. C. R., Caldas-Bussiere M. C., *Martins* R. S.Q. S., Menn-Barreto L. S and Cury L. J., (2007). Effect of exercise on occurrence of *diurnal rhythms of plasma* ions and metabolites in thoroughbred racehorses. *Arg. Bras. Med. Vet. Zootec*. 59, 857-861.

Vijayan M. M., Pereira C., Grau E.G and Iwama G.K., (1997). Metabolic responses associated with confinement stress in Tilapia: The role of cortisol. *Comp Biochem Physiol.116* (1) 89 – 95.

Vijayan M. M and Moon T. W., (1994). The stress–response and the plasma clearance of corticosteroid and glucose in a marine teleost, the Sea Raven. *Can J Zool*. **72**, 379-386.

Volpato G. L and Barreto R. E., (2001). Environmental blue light prevents stress in the fish Nile tilapia. *Braz JMed Biol Res.***34,** 1041-1045.

Volpato G. L and Fernandes M. O., (1994).Social control growth in fish. *Braz J Med Biol Res.***27**, 797-810.

Volpato G. L., Duarte C. R. A and Luchiari A. C., (2004). Environmental color affects Nile tilapia reproduction.*Braz JMed Bio Res.* **37**, 479-483.

Volpato G. L., Luchiari A. C., Duarte C. R. A., Barreto R. E and Ramanzini G. C., (2003). Eye color as an indicator of social rank in the fish Nile tilapia.*Braz JMed Biol Res.* **36**, 1659-1663.

Wendelaar-Bonga S. E., (1997). The stress response in fish. *Physiol Rev.***773**, 591-625.

WHO, (2006). Blood safety and clinical technology. Guidelines on standard operating procedures for clinical chemistry. Sodium and Potassium – Flame Photometry.

Wong, M., Tsukada, T., Ogawa, N., Pipil, S., Ozaki, H., Suzuki, Y., Iwasaki, W., & Takei, Y. (2017). A sodium binding system alleviates acute salt stress during seawater acclimation in eels. *Zoological letters*. **3**, 22.

Wright J. R. Jr., O" Hall W., Yang H., Han X and Bonen A., (1998). GLUT-4 Deficiency and severe peripheral resistance to insulin in the teleost fish tilapia. *Gen. and Comp. Endocrinol.***3**, 20-27

Wright J. R. Jr., Bonen A., Colnon J. M. and Pohajda B, (2015). Glucose Homeion B., (2015). Glucose homeiosa *Inter comparative glucose*. **40**(21), 234-23.

Yi Y., Lin C. K and Diana J. S., (1996). Influence of Nile tilapia (*Oreochromis niloticus*) stocking density in cages on their growth and yield in cages and ponds containing the cages. *Aquacul.***146**, 205-215.

Yong T., Walker S., Alfaro A., Fletcher L. M., Murray J. S., Lulijwa R and Symond J. E., (2019). Impact of acute handling stress anaesthesia on fish plasma biochemistry for veterinary screening and metabolomic sampling. *Fish Physiol Biochem*. **45**(4).

Zaghloul K. H., Omar W. A and Abo-Hegab S., (2005). Enviromental hazard risk assessment on; *Oreochromis nilotcus* and *Tilapia zilli* fish. *J Egypt. Ger.Sco. Zool*. **46**(A), 105-139.

Zhou X., Li M., Abbas K., and Wang W., (2009). Comparison of haematology and serum biochemistry of cultured and wild Dojo Loach. *Misgurnus anguillicaudatus*. *Fish Physiol biochem.***35**, 435-441.

APPENDICES

APPENDIX 1

Erythrocyte count protocol.

1. The Natt & Herrick`s stain solution and filter was inspected for any precipitates.

2. Whole anticoagulated blood samples is diluted using standard pipette and micropipettes with Natt & Herrick`s solution at the rate of 1:200.

3. Diluted sample is allowed to mix for 1 minute before discharging.

4. 10 µl of sample is discharged into the hemocytometer.

5. The samples` content is allowed 3 minutes to settle after charging into the hemocytometer.

6. Using a high dry (X40) objective lens of the microscope, total number of the RBCs that overlap the top and left border save for those that overlap the bottom or right borders, are taken for the sample in the four corners and central squares of the central large square (i.e., five red blood cell Squares) of the improved Neubauer counting chamber.

7. The total number of the RBCs counted is then multiplied by 10,000 to obtain the total erythrocyte count per microliter.

APPENDEX2

Cortisol ELISA Protocol (Neogen Corporation – USA).

TEST PROCEDURES

Scheme I: Preparation of standards:

Standard	Preparation
A	Stock solution 1µg/ml (was provided by the Neogen Corporation)
B	20 µl of A added to 980 µg of EIA buffer and vortexed = 20 ng/ml
C	200 µlof B added to 1.8 ml of EIA buffer and vortexed = 2 ng/ml
D	200 µl of C added to 1.8 ml of EIA buffer and vortexed = 0.2 ng/ml

Scheme1I: Standards dilutions

Standards	*ng/ml*	*EIA buffer (µl added)*	*B standards µl*	*C standards µl*	*D standards µl*
S_0	0.0	as is	-	-	-
S_1	0.04	800	-	-	200
S_2	0.0	500	-	-	500
S_3	0.2	-	-	-	as is
S_4	0.4	800	-	200	-
S_5	1.0	500	-	500	-
S_6	2.0	-	-	as is	-
S_7	10.0	500	500	-	-

2. The number of wells used was determined at 64. 16, 40 and 8 wells for standards, samples and blanks respectively.

3. Cortisol enzyme conjugate was then diluted by adding 1 µl of the enzyme conjugate into 50 µl total volume of EIA buffer for each well assayed.

4. 50 µl of standards (e.g. S_O, S_1....S_7) and samples (unknowns) (e.g.1, 2 ...20) was added to the appropriate wells in duplicate. (See, scheme II for the template design used for the assay).

5. 50 µl of the diluted enzyme conjugate was added to each well using multichannel (8 – channel) pipette for rapid addition.

6. The plate was gently tapped on the sides for effective mixing.

7. The plate was then covered with a laboratory plastic film from Pechiney Plastic Packaging Company – Chicago, USA and incubated at room temperature for 1 hour. This was done in a Lamina flow hood to avoid drafts and temperature fluctuations.

8. The concentrated wash buffer was diluted with deionized water (i.e. 20 ml of wash buffer plus 180 ml of deionized water) and mixed thoroughly.

9. After incubation, the content of the plate was dumped out. Thorough tapping out of contents on a clean lint-free towel then followed.

10. Each well was washed 3 times with 300µl of diluted wash buffer.

11. 150 µl of substrate was added to each well using multichannel pipette for best results and mixed by shaking plate gently.

12. The plate was then incubated in the dark at room temperature for 30 minutes.

13. The plate was gently shaken before taking readings to ensure uniform colour throughout each well.

14. The plate was read in a micro plate reader at 650 nm.

15. 8 wells were used as blanks with only substrate added into the wells (150 µl/well) for accounting for the substrate background. The average of the absorbance values of the blanks was subtracted from the absorbance values of the wells being assayed (samples wells).

Scheme III: ELISA Assay Template Design Used.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	S_0	S_0	B	B	Un_7	Un_7	Un_{15}	Un_{15}				
B	S_1	S_1	B	B	Un_8	Un_8	Un_{16}	Un_{16}				
C	S_2	S_2	Un_1	Un_1	Un_9	Un_9	Un_{17}	Un_{17}				
D	S_3	S_3	Un_2	Un_2	Un_{10}	Un_{10}	Un_{18}	Un_{18}				
E	S_4	S_4	Un_3	Un_3	Un_{11}	Un_{11}	Un_{19}	Un_{19}				
F	S_5	S_5	Un_4	Un_4	Un_{12}	Un_{12}	Un_{20}	Un_{20}				
G	S_6	S_6	Un_5	Un_5	Un_{13}	Un_{13}	B	B				
H	S_7	S_7	Un_6	Un_6	Un_{14}	Un_{14}	B	B				

S – standards, B – blanks, Un – unknown (samples), Un_1– Un10 HSD, Un_{10} – Un_{20} LSD.

APPENDIX 3

Standard Concentration (ng/ml), Optical Density (Absorbance Value) and %B/B_o.

Standard	Standard Concentration (ng/ml)	Optical Density (Absorbance Value)	%B/B_o
S_o (B_o)	0.0	0.764	100
S_1 (B_1)	0.04	0.655	86
S_2 (B_2)	0.10	0.627	82
S_3 (B_3)	0.2	0.439	57
S_4 (B_4)	0.4	0.427	56
S_5 (B_5)	1.0	0.290	38
S_6 (B_6)	2.0	0.252	33
S_7 (B_7)	10.0	0.226	30

APPENDIX 4.

Standard Concentration (ppm) and Optical Density/Absorbance value for Sodium, Potassium and Calcium ions.

Working standards (ppm)	**Optical density (Absorbance value)**		
	Na^+	**K^+**	**Ca^{++}**
0	0.0	0.0	0.0
2	2.8	1.7	2.0
4	4.1	4.0	4.0
6	6.7	6.1	6.0
8	8.8	8.1	8.0
10	10.4	10.5	10.0

APPENDIX 5

Flame photometry protocol

1. Plasma samples were first filtered using Whatman Filter Papers (7-18 cm diameter).

2. Samples were further filtered through Syringe filters.

3 Commercial Flame Photometer Standards (1000 ppm K, 1000 ppm Na and 1000 ppm Ca) were used to make calibration stock solutions by diluting and topping up 1 ml of each of the standards to 100 ml using distilled water in labeled 100 ml volumetric flasks.

3. The stock solutions were then used to prepare working standards for sodium, potassium and calcium in clearly labeled test tubes.

4. Each working standard solution was run one by one for these minerals and their absorbance values recorded. Each plasma sample was diluted 1: 5 with distilled water by mixing 1ml sample with 5 ml distilled water for Na^{+} , K^{+} and Ca^{++} concentration assays.