WHOLE TRANSCRIPTOME ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES IN NILE TILAPIA (*Oreochromis niloticus*) SUBJECTED TO CHRONIC STRESS

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A thesis submitted in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy in Molecular Biology of Masinde Muliro University of Science and Technology

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DECLARATION

This thesis is my original work, prepared with no other than the indicated sources and support and has not been presented elsewhere for a degree or any other award(s)

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CERTIFICATION

The undersigned certify that, we have read and hereby recommend for acceptance of, Masinde Muliro University of Science and Technology, a thesis entitled: **"**Whole transcriptome analysis of differentially expressed genes in Nile tilapia (*Oreochromis niloticus*) subjected to chronic stress"

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DEDICATION

This work is dedicated to my family: my dear wife Agnes, my daughters Hope and Esther, my son Levis and my nephew Levi. You gave up your comfort to stick by my side during the research.

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ABSTRACT

Chronic stress is the long-term activation of the stress response system and is a major bottleneck to aquaculture production as it lowers productivity and compromises fish welfare. Several studies have attempted to infer the presence of stress, however, there is paucity of information regarding quantification and the mechanisms by which chronic stress depresses growth. A few studies have attempted to determine which genes are regulated in chronic stress and how chronic stress impacts metabolic pathways. Furthermore, in cultivated Nile tilapia (*Oreochromis niloticus* L.), only a small number of genes conferring advantageous phenotypes have been identified. The current study investigated the relationship between stress levels and growth performance in relation to the metabolic pathways regulated in response to chronic stress in cultured Nile tilapia. Juvenile Nile tilapia were cultured in the laboratory at different ammonia concentrations and stocking densities for 70 days. Growth performance was determined alongside renowned stress markers: glucose and cortisol levels, followed by RNA sequencing and differential gene expression. Fish in the treatment groups showed negative allometry while the controls showed positive allometric growth. The specific condition factor (K_n) ranged from 1.17 for the controls to 0.93 for the ammonia treatment and 0.91 for the stocking density treatment. Results of this study indicated a positive correlation between the levels of stressors and the indicators of stress i.e. concentrations of blood glucose, plasma cortisol and scale cortisol. There was a significant difference ($p<0.05$) in the mean plasma cortisol levels between ammonia treatments and the control ($p< 0.05$ i. e 4.71 ± 0.52 ng/ml and 6.50 ± 0.83 ng/ml) respectively. The cortisol levels increased concomitantly with the concentration of ammonia. There was also a significant difference in the plasma cortisol levels between the low fish stocking densities and the high fish stocking densities. Comparative transcriptome analysis revealed 209 Differentially Expressed Genes (DEGs) (156 up- and 53 down-regulated) in ammonia and 252 DEGs (175 up- and 77 down-regulated) in stocking density treatment. In both treatments, 24 and 17 common DEGs were up- and down-regulated respectively. DEGs were significantly enriched in six pathways associated with muscle activity, energy mobilization and immunity. The heightened muscular activity consumes energy which would otherwise have been utilized for growth. Comparative genomics identified similarities between fishes with common genetic and evolutionary ancestry, allowing for better adaptation to local environmental conditions. Some of the selected genes exhibiting substantial effect on immunity include: *Prxs, MMR1 like, ZMYM4-like partial*; Stress reactive genes including: *PALLD-like* gene, *LPLBAG6-like* and growth-related genes including: *NF1x like*, *PEDF* and *CL like*. Experimental sample, *O. niloticus, O. aureas and Danio rerio* can hybridize in their natural environments bringing about genetic admixture ancestry that hybridises new genes which confer beneficial phenotypes. These results bring to fore the molecular mechanisms underlying chronic stress' suppression of growth in cultured Nile tilapia and can inform formulation of breeding programmes targeting stress resistance. The results of this study lays the foundation for the development of fish breeds that are climate-ready and able to weather climate shocks. This will allow Aquaculture to contribute to food and nutrition security in line with SDG2 and improve the economic status of fish farming communities in the Global South.

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Abca1	ATP binding cassata transporter a1
ADP	Adenosine Di-Phosphate
ANOVA	Ananlysis of Variance
ATP	Adenosine Tri-Phosphate
BCL2a	B-cell Lymphoma 2a
BP	Biological Process
CC	Celluar Component
CNS	Central Nervous System
CRISPR-Cas9	Clustered Regulatory interspaced short palindromic repeats and CRISPR- associated protein 9.
CTS-L	Cathepsin L like
DEGs	Differentially Expressed Genes
DNA	DeoxyRibonucleic Acid
DO	Disease Ontology
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agriculture Organisation of the United Nations
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
GIFT	Genetically Improved Farm Tilapia
Glut	Glucose Membrane Transporter
GO	Gene Ontology
HRP	Horseradish peroxidase
IL-6	Interleukin 6
IL-8	Interleukin 8
ISERC	Institutional Scientific Ethics Review Committee
KCSAP	Kenya Climate Smart Agriculture Technology
KEGG	Kyoto Encyclopedia of Genes and Genomes
LPLBAG6	Large Proline rich protein BAG6 like
LWR	Length Weight Relationship
MAPK	Mitogen Activated Protein Kinase

MF	Molecular Function
MMR1	Macrophage Mannose Receptor 1
MMUST	Masinde Muliro University of Science and Technology
MMUST	Masinde Murilo University of Science and Technology
Mylk3	Myosin light chain kinase 3
NACOSTI	National Commission for Science, Technology and Innovation
NCBI	National Center for Biotechnology Information
NCBI	National Center for Biotechnology Information.
NF1X	Nuclear Factor 1-x type
NF-kb	Nuclear Factor kappa B
PALLD	Palladin like
PAMM	Peroxiredoxin Activated in M-CSF stimulated Monocytes
PAV	Presence and Absence Variants
PEDF	pigment epithelium derived factor
PPT	Parts Per Thousands
PRXS	Peroxiredoxin
RNA	Ribonucleic Acid
RSK	Ribosomal s6 Kinase
RT-qPCR	Real Time quantitative polymerase chain reaction
SDG	Sustainable Development Goals
SGR	Specific Growth Rate
Slc12	Solute carrier family 12 member
Slc25a24	Solute carrier family 25 member 24
SNPs	Single Nucleotide Polymorphism
SOFIA	State of the World Fisheries
SPSS	Statistical Package for Social Sciences
SRAC	Southern Region Aquaculture Center
TMB	3,3'.5,5' Tetramethyl Benzidine
UIA	Unionised of Ammonia
VASP	Vasodilator Stimulated Phosphoprotein
ZMYM4	Zinc Finger MYM-Type Containing 4

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Fish is increasingly becoming an important source of safe nutritious food rich in amino acids, unsaturated fatty acids, vitamins, and trace metals. Fish based dietis a rich source of proteins, essential omega -3 amino acids and bioavailable micro-nutrients (FAO, 2022). Aquaculture intensification is on the rise driven by increasing demand for fish and fisheries products coupled with the decline in capture fisheries (FAO, 2022). There is pressure to produce more fish per less area in order to feed the ever-growing world population (Rodriguez-Barreto, 2019). Unfortunately, this pressure has often resulted in farming more fish in limited space leading to overcrowding and overstretching water environments. Additionally, this production pressure has resulted in the culturing of fish in environments that they are not traditionally adapted to (Johnson *et al*., 2014). This causes either acute or chronic stress in fish. The stress ensuing from crowded sub-optimal water environments lowers the productivity and compromises fish welfare (Martos-Sitcha *et al*., 2020). Tilapia "the aquaculture chicken" is reputed as one of the fastest growing fish and with very good adaptability to wide range of environmental conditions. Tilapia has gained ground becoming the second most cultured fish species in the world after carps due to ease of culture, mild taste and profitability (Wang and Lu, 2015). Tilapia is omnivore , has a good tolerance to high stocking densities and handling, good growth on natural feeds, a variety of supplemental feeds (manufactured and by-products) and is relatively tolerant to poor water quality and diseases (El-Sebai *et al*., 2018). Tilapia is native to Africa but has been introduced and is now cultured in more than 140 countries in the world with a market value

of 12 Billion US dollars which is expected to grow to 25 Billion by 2028 (FAO, 2018). China is by far the biggest producer of tilapia in the world with an annual production of over 1.749 millon tonnes (FAO, 2018). In Kenya, tilapia aquaculture makes up to 75% while cat fish makes up 18% of the total aquaculture production. Most of the farmed tilapia in Kenya is produced from fresh water earthen ponds and grown in static water (Opiyo *et al.,* 2018).

There are many fish breeding programmes that have been set up targeting mainly increase in productivity. The genetically improved farm tilapia (GIFT) has continuously been improved with each generation growing between 5 - 10 % faster than the previous generation (World fish, 2016). In the wild different fish species live in the same environment. There is tangible evidence that some of these fish can interbreed to produce an offspring of mixed ancestry and in the process acquire and transmit genes that are not traditionally found in them (Angienda *et al* 2011). Some of these introgressed genes confer beneficial phenotypes that enable the offspring to be better adapted to varying culture environments. This hybridization is the basis of fish breeding programs. Most breeding programs have laid major focus on genetic improvement that has the capacity to deliver cumulative and sustained improvements in production efficiency, product quality and ultimately, financial profitability of aquaculture enterprises and industries (Krawchenko and Stoney, 2011).

However, there is paucity of information regarding breeding for environmental resistance. This study sought to establish the relationship between environmental conditions, growth performance and gene expression. Particularly this study focused on the influence of

ammonia and stocking density on gene expression in cultured Nile tilapia (*Oreochromis niloticus*).

The use of genomic tools can enable the study of candidate genes that are associated with stress responses (Bahji *et al.,* 2021). These genes can be used as unique signatures for specific stressors and could be used to mark for early signs of stress onset. On the other hand, these marker genes can be introgressed to confer the advantageous phenotypes into the cultured fish. Ammonia is one of the most toxic substances known to cause growth inhibition and fish mortality in aquaculture. The levels of ammonia in the fish body is directly related to its levels in the culture media. Many studies have laid emphasis on the devastating effects of ammonia concentration spikes associated with high levels of mortality in fish especially in intensive culture systems and during periods of crowding together e.g. during transportation. There is paucity of information on the "sub lethal" effect of ammonia in production systems (Randall and Tsui, 2002). The effect of sustained or increasing levels of ammonia in growth media and fish growth performance, gene expression as well as fish metabolic processes remains largely un-documented.

Crowding is a common practice in aquaculture (Abdel Tawwab *et al.,* 2014). Farmers are increasingly being compelled to produce more in smaller areas leading to crowded conditions. However, optimizing fish population and fish production often compromises fish welfare. Tilapia is known to be a solitary fish but at higher stocking densities, it changes from antagonistic (aggressive) to shaoling behavior (Rodriguez-Barreto, 2019). Crowding has been demonstrated to cause social stress in Nile tilapia bringing about a shift from a proactive to a reactive stress coping style (Champneys *et al.,* 2018). Information

about the effect of crowding on growth performance and gene expression in relation to the metabolic processes remains scanty.

1.2 Justification of the Study

Aquaculture is characterized by fish held at high stocking densities (Li et al 2021). While the effect of this high-density confinement of fish has been extensively studied, its importance in balancing between productivity and fish welfare is not properly documented. It is important to understand fish stress physiology at the molecular level to gain useful insights in fish stress resistance and probably come up with new selection methods that could take into account the fish welfare - productivity balance. To meet the ever increasing demand for fishery products, more fish will have to be farmed in smaller areas. This therefore means that since crowding has been demonstrated to be stressful to many fish species, selection of individuals that are more suited for high density confinement is apparent.

Tilapia aquaculture is practiced in many systems ranging from extensive, semi intensive to highly intensive systems. However, the more profitable intensive systems are expensive to set up and run due to the cost of maintaining good water quality. High economic losses may occur when there is failure of the water management system even for short durations of time. It is therefore desirable to choose fish that are more resistant to the stress occasioned by poor water quality. In pond aquaculture, there is continuous addition of feeds and accumulation of fecal matter with time. These two are the major contributors to increased ammonia in fish ponds. Since tilapia is usually farmed in static water, there is a risk of ammonia levels rising to toxic levels with time. Ammonia has been shown to cause stress in fish and result in poor feed intake, reduced disease resistance and depressed

growth. Abdalla and Heba (2011) showed that when tilapia is subjected to high levels of ammonia, some easily succumbed while others survived. So far there is no documentation of the molecular basis of this apparent resistance. It is therefore important to determine the genes expressed during prolonged stress. There is need to establish the relationship between stress levels, gene expression and growth performance in farmed fish.

There is an increased demand for the production of more fish protein for the ever increasing global population within the limitation of resources (Rodriguez- Barreto 2019). This therefore implies using less areas to farm more fish leading to production of fish in overcrowded environments. It is therefore important to select fish that can perform well in crowded spaces without adversely affecting their growth performance. This therefore calls for understanding of the coping abilities ingrained in these fish enabling them to escape the stress thereof. While there are some studies detailing effects of stress on gene expression, there is no documented account of the link between stress levels, gene expression and fish productivity. This present study seeks to find the relationship between the gene expression and productivity in cultured Nile tilapia reared in crowded environments and in high ammonia environment.

The outcome of this study can help to inform the designing of breeding programmes targeting stress resistance and growth performance in fish. The findings from this study will also help inform policy on fish welfare management, covering a range of biological and environmental factors, which is a key component in enhancing aquaculture productivity.

1.3 Statement of the Problem

Unlike chronic stress, the effect of acute stress on growth performance in fish has received a lot of interest. This may be because acute stress leads to high levels of fish mortalities in the aquaculture establishments. On the other hand, despite chronic stress being mild and thus not causing mortalities, the loss in productivity leads to huge economic losses in the aquaculture industry. The chief causes of chronic stress in aquaculture are ammonia and high stocking densities. Sources of ammonia in aquaculture systems are ammonification of organic matter such as the uneaten fish feeds and other decomposing organic matter, fish fecal matter and fertilizers. On the other hand intensification practices have led to increased stocking densities in culture systems. Chronic stress has been shown to depress the growth rate of fish and is therefore a bottle neck in aquaculture production. There is paucity of information about the effect of the magnitude of the chronic stressor on growth performance in fish. While high ammonia concentrations have been shown to be stressful to fish, data on the optimum concentration beyond which stress occurs is lacking. Chronic stress has been shown to influence the gene expression in fish. However, there is limited information on the relationship between growth performance and gene expression in cultured Nile tilapia. Fish responds to stress by regulating metabolic pathways in a way to enable it to cope with the stressors. However, information regarding the identity of these pathways remains scanty and poorly documented. As a result of their genetic makeup, some fish have the ability to survive in stressful environments. Hybridization of fish with stress resistance genes with those lacking these gens has been demonstrated to bring forth an offspring with superior stress resistance abilities compared to their susceptible parent.

Selection of these desirable genes forms a foundation for breeding programs aimed at improving stress resistance in cultured fish.

1.4 Significance of the Study

It is necessary to demonstrate a relationship between stress levels, gene expression, and growth performance in farmed fish. This study's findings can be utilized to help develop breeding programs for stress tolerance and growth performance in fish. The study will expand the number of stress markers available in fish. The findings will be useful in future research on chronic stress in fish and other species. The outcomes of the study will help impact policies on fish welfare management, which is an important component in enhancing aquaculture productivity.

1.5 Objectives of the Study

1.5.1 General Objective

To characterize the entire transcriptome profile of differentially expressed genes in cultured Nile tilapia (*O. niloticus*) subjected to chronic stress and its association with growth performance.

1.5.2 Specific Objectives

i. To determine the levels of growth performance of cultured Nile tilapia (*O. niloticus*) under different ammonia concentrations and stocking densities.

ii. To determine the ammonia and stocking density induced stress in cultured Nile tilapia (*O. niloticus*).

iii. To determine the whole transcriptome analysis of cultured Nile tilapia (*O. niloticus*) subjected to chronic ammonia and stocking density stress.

iv. To undertake a comparative pangenome analysis of the whole genome sequence script of the experimental sample and map it to a reference genome in the database.

1.6 Hypothesis

i. **H01:** There is no variation in the level of growth performance of cultured Nile tilapia (*O. niloticus*) under different ammonia concentrations and stocking densities.

ii. **H02:** There is no variation in the level of stress in cultured Nile tilapia (*O. niloticus*) under different ammonia concentrations and stocking densities.

iii. **H03:** There is no difference in the whole transcriptome of cultured Nile tilapia (*O. niloticus*) subjected to chronic ammonia and stocking density stress and their controls.

iv. **H04:** There are no significant difference between the genome sequence of the experimental sample and the reference genome in the database.

CHAPTER TWO

LITERATURE REVIEW

2.1: Introduction

Fish is a major diet component and contributes 17% of animal protein, and 7% of all proteins consumed world over and is the major protein source for over 3 billion people in developing countries (Obiero *et al*., 2019) and employs 59.6 million people on a full-time, part-time or occasional basis (FAO, 2018). Fish consumption has numerous health and nutritional benefits such as being rich in amino acids, unsaturated fatty acids, vitamins, and trace metals (Simopoulos 2000). Fish consumption has been growing steadily over the last twenty years (FAO, 2018) with the global per capita consumption increasing from 9.0 kg in 1961 to 20.3 kg in 2017 (FAO, 2020). Coupled with the population growth, the demand for fish has risen from 143.8 million tonnes per year in 2011 (European Commission's Joint Research Centre (JRC)) to 156.4 million tonnes per year in 2018 (FAO 2020). Capture fisheries can no longer keep pace with the increasing demand for fishery products. Furthermore, the World Capture fisheries have levelled off with most capture fisheries having already been fully exploited. The advancement in the fishing technologies used, destruction of breeding grounds and habitat loss, weak government policies, inadequate stakeholder involvement, environmental pollution, and climate change are some of the factors that majorly contribute to the exploitation of the capture fisheries leading to the dwindling catches. This decline is happening in the background of an increasing demand for fish and fisheries products (Figure1).

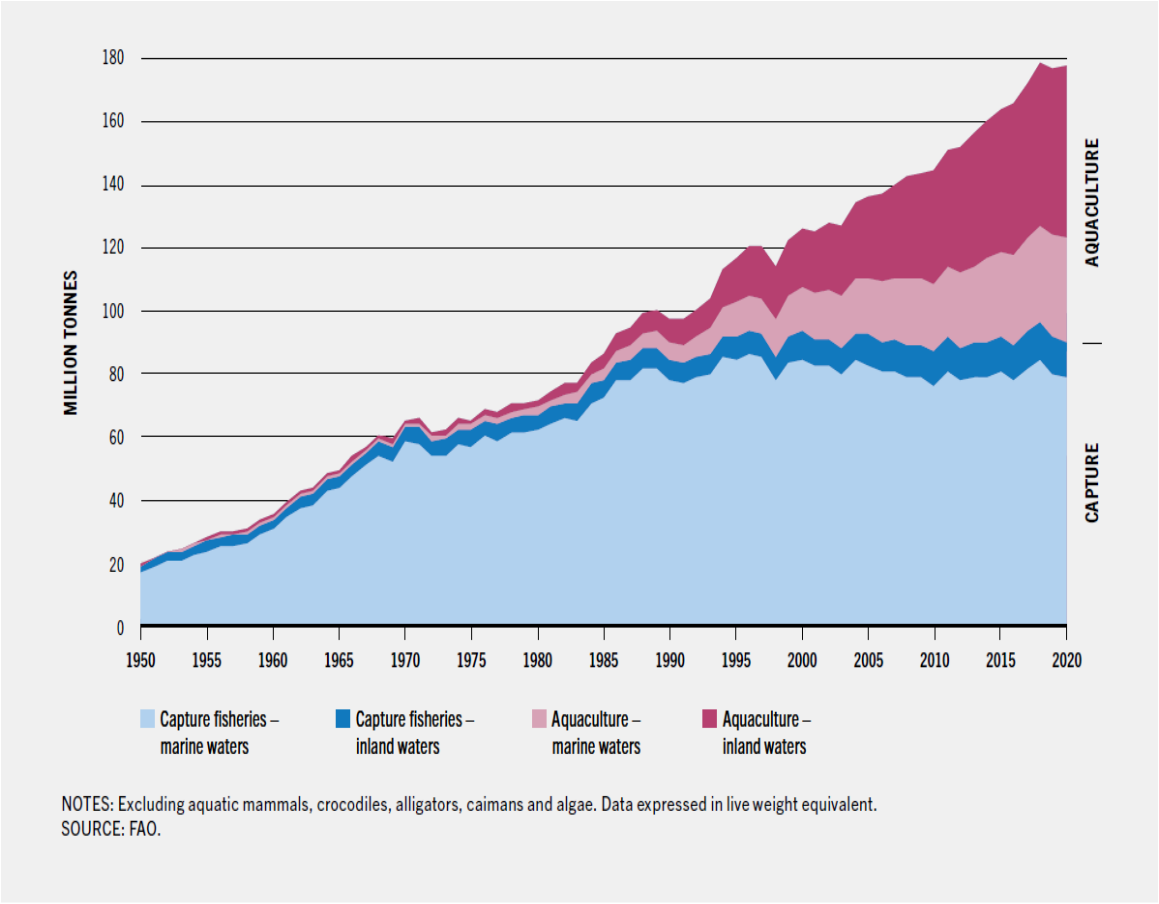

Figure 1: World capture fisheries and aquaculture statistics for the years 1950-2020: Adopted from the State of World Fisheries and Aquaculture 2022 (FAO 2022).

Aquaculture production is therefore the alternative to breach the gap between production and consumption (Prabu *et al*., 2019). Globally, Aquaculture has been on the upward trend (FAO. 2018) with production doubling every decade for the last 50 years (Bostock *et al.*, 2010). Aquaculture has now become the major fish production technology with the capacity to meet the rising demand of fishery products (Golden *et al*., 2017). Aquaculture is currently the fastest growing food production sector in the world fanned by advances in fish genetics, nutritious feeds, culture systems, and management (Small *et al*., 2016). The aquaculture production of tilapia has quadrupled in the last decade making it the second most farmed fish worldwide (Prabu *et al.*, 2019). Statistics show that aquaculture

production has now overtaken capture fisheries (FAO, 2022) and continues to increase in significance with time (Hannah and Max, 2020; Small *et al*., 2016; Belton and Little, 2011) and is projected to increase to 62% by 2030 (FAO, 2014). The total fish supply is projected to increase from 179 million tons in 2018 to 204 million tons in 2030, with aquaculture being entirely responsible for the increase (FAO, 2020; Kobayashi *et al*., 2015). Aquaculture production is expected to reach 109 million tonnes by the year 2030 with the fastest growth expected for tilapia and shrimps.

2.2 Aquaculture Production Technologies

Aquaculture aims to increase production and productivity, which necessitates some type of rearing process intervention. In the rapidly expanding field of aquaculture, farmers are increasingly under pressure to produce in an optimal, sustainable and animal friendly way (Bush *et al.*, 2013). This production pressure necessitates the farmer to manipulate the production process in an attempt to increase production in the limited spaces available. Some of the factors that are highly manipulated include stocking densities, feeds, feeding and genetics (Small *et al*., 2016). The fish culture environment deteriorates as a result of these manipulative techniques. Fish like any other animal gets stressed when the environmental conditions are unfavorable e.g., extremes of temperature, pH, salinity, oxygen, carbon IV oxide, turbidity, water hardness and presence of predators. The resultant stress has been shown to affect fish growth and survival. Other practices carried out in fish farming like handling and exposure to atmospheric oxygen during sampling, sorting and transporting exposes fish to stress. Crowding, reducing the water levels, and increasing the fish stocking density are common husbandry practices in aquaculture that may also increase stress in cultured fish (Abdel-Tawwab *et al*

2014). Farmers frequently overstock and overfeed their fish since aquaculture is a profit-driven industry, with the goal of maximizing productivity. This can occasionally lead to a decline in water quality, which can cause fish mortality and impact farm output and profitability. Therefore, to maintain the water quality required for the healthy development of fish, it is imperative that a fish farmer strike the correct balance between intensification and the applicable management approaches.

2.3 Aquaculture Production Systems

Fish farming is usually carried out using either extensive, semi-intensive or intensive systems depending on the level of investment and stocking densities. In Kenya, most fish farmers use the earthen pond semi-intensive culture system (Opiyo *et al.,* 2018). This system is characterized by low stocking densities and minimal application of supplementary feeds. The requirement for monitoring of the water quality parameters in this culture system is less stringent when compared to intensive systems (Mohanty *et al.,* 2018). Semi intensive tilapia production happens in static green water fertilized by addition of organic manure and / or inorganic manure. Kenyan aquaculture is dominated by tilapia produced in semi-intensive systems (Opiyo *et al.,* 2018). There is however a growing need to intensify and graduate farmers from this semi-intensive to intensive aquaculture systems which are more commercially oriented, takes up less space and requires less water. Such is spurred by the increasing demand of aquaculture products and the increased aquaculture production know how on the part of the farmers. Several support programs targeting this category of farmers have been rolled out in Kenya in the recent past such as the Aquaculture Economic Stimulus program (AESP), Kenya Climate Smart Agriculture project (KCSAP) and the Aquaculture Business Development Program (ABDP). With increase in the level

of intensification, the use of commercial feeds and fertilizers will increase leading to an increase in the nitrogen load in the pond.

2.3.1 Ammonia in Aquaculture Systems

Ammonia is very toxic to the body and has detrimental effects on many different organs and organ systems of many animals. Ammonia is an important water quality parameter that has a direct impact on the behavior, health and growth performance of fish in ponds tanks and aquariums. Under poor aquaculture conditions, ammonia has been identified as one of the main causes of mass mortality in fish. (Ip and Chew 2010). Dissolved oxygen and pH have been shown to directly influence the toxicity of ammonia in aquaculture systems. When the temperature and pH are high, toxicity due to ammonia increases (Shin *et al*., 2016, Patra *et al.,* 2015). In aquaculture systems, ammonia exists in two forms that are in a dynamic equilibrium: the ionized (NH_4^+) and the un-ionised (NH_3) forms. The un-ionised form of ammonia (UIA) is more toxic to fish due to its higher ability to diffuse through the gills (Sinha *et al.,* 2012) into the body of the fish. The higher toxicity associated with increased temperature and pH is due the conversion of ionised to the un-ionised form. Toxicity of ammonia begins at as low as 0.05 mg/L with fish beginning to die at 2.0 mg/L of UIA.

The chief sources of ammonia in fish ponds are ammonification of organic matter (Paul *et al.,* 2020) such as the uneaten fish feeds and other decomposing organic matter, fish fecal matter (Zhou and Boyd 2015; Crab *et al*., 2012) and fertilizers (Claude, 2018). Significant amounts of ammonia nitrogen are added to fish culture systems in many places by the widespread use of nitrogenous fertilizers, particularly ammonium chloride, and urea, which enter aquaculture water with the washouts of rainwater and surface runoff. Domestic

sewage discharge and industrial wastewaters are two additional sources of ammonia in aquaculture systems. Increasing stocking density levels results in increase in nitrogenous wastes excrement thus increasing the levels of ammonia in the fishpond. Ammonia is the major product of protein catabolism in fish and constitutes 60% to 80% of the total nitrogenous wastes (Aysel and Gluten, 2005). The undigested nitrogenous waste in fish fecal matter together with the uneaten feeds tends to settle at the pond bottom and slowly undergo decomposition resulting in production of more ammonia (Paul *et al.,* 2020). However, nitrification, an opposite process converting ammonia to nitrates also takes place at the pond bottom. Nitrification occurs through bacterial assisted processes where ammonia is converted to nitrates via nitrite. While nitrate is less toxic to fish, excess nitrates causes proliferation of algae which in turn result in anoxic environment for fish. Removal of nitrates is done by denitrifying bacteria that converts nitrates into atmospheric nitrogen gas. The process of denitrification is however slow especially because of the anoxic environment of the pond bottom sediment (Avnimelech, 2003). The decomposition of nitrogen containing fish feeds and fecal matter is quite rapid compared to the denitrification process resulting in accumulation of ammonia in fish ponds. Besides the denitrifying bacteria, ammonia in fish ponds is subjected to natural restriction by absorption by phytoplanktons, removal via water exchange, adsorption to the cationic sites on the pond bottom soils and when pH is high some ammonia is lost via diffusion into the air especially during windy days and in highly aerated ponds. The rate of excretion of ammonia by fish is directly related to the protein content in the feed used as well as the amount of the feed used (Li *et al.,* 2015). Fish feeds usually contains a higher level of protein compared to those of other domestic animals. Many times fish farmers are tempted to be more than

generous when feeding their fish. Overfeeding and over stocking combined would therefore increase the risk posed by ammonia tremendously.

2.3.2 Ammonia Toxicity

Ammonia exposure has been found to cause convulsions, unconsciousness, and finally death in fish and other animals by causing oxidative stress, neurotoxicity, tissue damage, and lowered immunity (Ip and Chew 2010; Xu *et al*., 2021). This may be associated with the displacement of potassium ions by ammonium resulting in depolarization of neurons leading to activation of N-Methyl-D-Aspartate (NMAD) type glutamate receptor. The sodium Na^+/K^+ ATPase pump is activated by NMAD, accelerating the energy depression that finally results in cell death in the brain. Additionally, NMAD has been linked to both intracranial hypertension and mortality by causing cerebral edema by releasing glucose (Xu *et al*., 2021). Once activated the receptor allows an influx of excess calcium ions into the CNS which causes cell death (Randall and Tsui 2002). Stressed fish produces more ammonia and are more sensitive to ammonia toxicity from the external environment.

High levels of ammonia have been associated with depressed growth performance, poor feed conversion, decreased reproductive capacity, depressed growth rate (Zhou et al., 2020) and reduced disease resistance in fish (SRAC, 2018). According to El-Shafai *et al.*, (2004), ammonia is one of the most toxic substances known to cause growth inhibition and fish mortality in aquaculture. Ammonia at concentrations above 0.3 mg/L has been shown to cause death to some fresh water fish (Pearson-Le Ruyet *et al*., 1995). Exposure to ammonia has been shown to cause alterations in blood parameters such as red blood cell (RBC) counts, hemoglobin, and hematocrit of African catfish, *Clarias gariepinus*, exposed to ammonia (Ajani 2012). Due to its strong affinity for hemoglobin, ammonia inhibits the

blood's ability to transport oxygen and can thereby replace it. Similar findings were noted in common carp, *Cyprinus carpio (*Thangam *et al*., 2014), rockfish, *Sebaste schlegelii* (Shin *et al.*, 2016), Atlantic Salmon, *Salmo salar*, (Knoph and Thorud,1996). Shin *et al.*, 2016 reported significant alterations in serum components (total protein, glucose, serum glutamic oxaloacetic transaminase (GOT) and serum glutamic pyruvic transaminase (GPT) in rock fish exposed to ammonia. Channel Catfish exposed to 0.52 mg/L ammonia have been shown to grow at a rate that is 50% lower than the unexposed controls (Schlenk, 2000).

High environmental pH, especially when the buffering capacity of the pond water is low (Weihrauch *et al*., 2009), reduces the gradient for NH_3 diffusion leading to a build up of ammonia inside the fish (Yuen and Shit, 2010 ; Lemarie *et al*., 2004; Li *et al*., 2014). Increased concentrations of ammonia in the pond water has been shown to induce neurotoxicity, oxidative stress, oxygen delivery impairment as well as hyperactivity, convulsions and coma (Wilkie 1997) which may eventually lead to fish death.

Un-ionized ammonia is able to cross the gill epithelium through passive diffusion in a process aided by rhesus (Rh) glycoproteins. When exogeneous ammonia is high, diffusion of ammonia from the fish body is impaired and if the levels are sufficiently high, ammonia can cross into the fish body via the same route (McKenzie *et al*., 2009; Randall and Tsui 2002). Elevated levels of ammonia in the water leads to accumulation of ammonia in the fish body (McKenzie *et al*. 2009). Toxicity of ammonia is mainly due to the un-ionised form however, at high levels, NH_4^+ has also been shown to cause toxicity to fish (Silvia *et al*., 2013).

2.4 Crowding in Aquaculture

Stocking density is a major factor influencing the profitability and economic sustainability of an aquaculture establishment (Aidos *et al*., 2020). In trying to increase productivity fish farmers tend to increase the stocking density which in turn increases the occurrence of chronic stress to the fish (Aidos *et al*., 2020; Iguchi *et al*., 2003). Crowding has been identified as a common chronic stressor in fish that has the potential to depress fish growth (Abdel-Tawwab 2014). High stocking density has been shown to negatively affect both fish growth and feed utilization (Ridha, 2006; Dediu *et al*., 2021). High stocking densities have been shown to reduce feeding activity and growth rates in Coho Salmon (*Oncorhynchus kisutch*), and rainbow trout (*Oncorhynchus mykiss*). This phenomenon has also been demonstrated in Siberian Sturgeon (*Acipenser baerii*) larvae (Aidos *et al*., 2020). Reduced growth in fish reared at high density may be due to lower feed intake, adverse social interactions (Ellis *et al*., 2002; Naderi *et al*., 2017) and the mobilization of dietary energy by the physiological alterations provoked by the ensuing stress (El-Sayed, 2002). It has been shown that crowding stress negatively affect *O. niloticus* survival, percentage weight gains and specific growth rate in fry (El-Sayed, 2002) and growth performance in adults (Ridha, 2006).

High stocking density causes a shift in association behavior of tilapia from the antagonistic (aggressive) to shoaling (Gall and Bakar, 1999) and their stress coping mechanisms shifts from proactive to reactive (Champeneys *et al*., 2018). Montero *et al*. (1999) demonstrated that high stocking density produced a chronic stress situation in gilthead seabream, *Sparus aurata* juveniles. This study also showed that crowding brought about a four-fold increase in plasma cortisol levels, haemoconcentration, decrease in alternative complement

pathway and an altered liver fatty acid composition. Crowding was also reported to influence lipid distribution and immune function in gilthead seabream juveniles. A four-fold increase in whole body cortisol levels was also demonstrated in zebra fish held in crowded state for 3 hrs. (acute stress) and 5 days (chronic stress) (Ramsay *et al*., 2009). High stocking densities has been shown to produce a wide variety of effects on cultured fish populations, such as alterations in behavior, feed utilization, resulting in poor growth, immune functions, intestinal microbiota and disease resistance and sometimes even mortality (Telli *et al*., 2014; Ribeiro *et al*., 2015). Crowding has also been reported to cause stress that lowers the immune response in fancy carp (*Cyprinus carpio* L.) where the activities of non-specific defense mechanisms were lowered (Yin *et al*., 1995).

2.5 Stress in Fish

Stress is a physiological response of an organism to a threatening situation whether real or imaginary. Stressors can be intrinsic or extrinsic stimuli that threaten to disturb the dynamic equilibrium of an animal organization called homeostasis (Wendelaar-Bonga, 1997). Some of the major causes of stress to fish in ponds includes; alteration of the physical and chemical characteristics of the water environment, poor fish handling methods, harassment from other fish and predators, overcrowding and poor nutrition (Aidos *et al*., 2020; Odhiambo *et al*., 2020). Many farm management practices can also induce stress in fish such as sampling, handling, exposure to air, grading and transportation.

The water environment is very important to the fish since the fish lives, feeds, excretes, respires and reproduces in the water. Alteration of water quality thus has a high impact on the live of the fish and is a major source of stress in fish. Some of the water quality parameters that have significant impact on the fish wellbeing includes; ammonia levels,

elevated nitrates levels, extreme pH, low oxygen levels and extremes of temperature. Exposure of fish to such conditions has been shown to trigger stress reaction either acute or chronic depending on the severity and duration of exposure (Odhiambo *et al.,* 2020)

Prolonged exposures to stress negatively affects other necessary life functions in fish such as growth, development, disease resistance, behavior, and reproduction (Schreck and Tort 2016). Stress response involves the reorganization of the fish's energy budget, immune system and endocrine mechanisms to cope with the environmental challenge (Flik *et al*., 2018). Fish stress has been demonstrated to be a major cause of disease outbreaks in the pond environment (Rottmann *et al*., 1992). Thus Stress directly influences productivity and its management is paramount if a farmer is to increase profitability of a fish farming venture. Furthermore, it has been shown that a stressed fish can influence the stress levels of conspecifics (Barcellos *et al.,* 2011) hence the need to always maintain as stress free environment as possible.

2.6 Effects of stress

Numerous changes brought about by the stress reaction may have a detrimental impact on how well farm animal's function. Among these impacts include changes in immune system functioning, an increase in illness susceptibility, a decrease in feed consumption, lower fertility, decreased growth performance, increased mortality rates and decreased productivity. Somatic growth has been shown to reduce significantly as a result of exposure to chronic stress (Carnosa and Bertucci2023). Research has shown that Innate and adaptive immune responses are dysregulated or suppressed by long-term stress (Dhabhar 2014). Stress has also been shown to negatively affect the feeding habits of fish and therefore lowering their productivity and increasing their mortality rates (Abd El-Hack

et al., 2022). Stress not only affects the quality of life of an organism but also the product quality and quantity produced by such stressed animals. In human beings, it has been shown that stress in new mothers affects the quality and quantity of milk produced (Foligno *et al*., 2020). Heat stress has been reported to affect meat and egg quality as well as egg weight in chicken (Lara 2013; Oyewole *et al.,* 2016). In fish stress has been associated with poor fillet quality and yields (Daskalova 2019)

2.6.1 Stress and Fish Flesh Quality

Pre-slaughter stress has been shown to affect the quality of meat in many animals including fish (Daskaalova, 2019). Muscle glycogen is the main source of energy in healthy unstressed animals. Stress metabolism results in depletion of glycogen leading to lower post mortem production of lactic acid in the muscles. Lactic acid is necessary to produce meat which is tasteful and tender, of good keeping quality and good color. During stress, there is altered metabolism resulting in decline in energy reserves in the body. Acute stress increases anaerobic glycolysis leading to production of lactic acid which in turn lowers the muscle pH (Poli *et al.,* 2005). Chronic stress on the other hand results in clearance of the lactic acid but the energy reserves were exhausted resulting in higher muscle pH. The exhaustion of ATP in the muscles of the fish leads to a faster onset followed by earlier resolution of rigor motis (Terlouw, 2008; Hamm 1977; Gregory and Grandin, 1998). In chronically stressed animals, rigor mortis would proceed without production of lactic acid thus the pH in the muscles will remain high, resulting in the absence of the pre-rigor phase and a full rigor without decreasing pH, called alkaline rigor mortis (Goes *et al.,* 2019). Onset and resolution of rigor motis is an important aspect in fish processing both in terms of fillet yield and quality (Poli *et al* ., 2005). Pre-rigor filleting is associated with slower

bacterial growth increasing shelf life of the fillet (Tobiassen *et al.,* 2006) and retention of some important meat quality traits such as colour, texture and water holding capacity (Daskaalova, 2019). Rapid loss of freshness has been demonstrated in Atlantic cod subjected to pre slaughter chasing and reduced water quality (Digre *et al*., 2011).

2.6.2 Stress Response in Fish

In many aspects, the stress response of teleost fish mirrors that of terrestrial vertebrates. These are related to the main messengers of the brain-sympathetic-chromaffin cell axis and the brain-pituitary-interrenal axis as well as the functions of these pathways, which include stimulation of oxygen uptake and transfer, mobilization of energy substrates, reallocation of energy away from growth and reproduction, and primarily suppressive effects on immune functions (Wendelaar-Bonga, 1997). When confronted with a stressful situation, fish will respond by activating two hormonal axis: the hypothalamo–pituitary–adrenal/interrenal axis (HPA/I) and the sympatho-chromaffin (SC) axis. These responses aims at enabling the fish to escape or overcome the stressor / stressful situation. The activation of the SC axis results in increased ventilation which is mediated by an increase in the heart rate, the heart stroke volume leading to faster supply of large volumes of blood to the gills and muscles delivering oxygen and glucose to critical muscles for the escape.

The HPI axis on the other hand is involved in the reallocation of resources to increase the supply of glucose in the blood by increasing catabolic pathways and depressing other energetically costly processes such as immune responses and reproduction. The HPI axis activation culminates in the release of glucocorticoids from interrenal cells located in the head kidney (Gans *et al*., 2021) leading to production of corticosteroids mainly cortisol (Aidos *et al.,* 2020). Cortisol has for long been used as the indicator of stress in fish.

Elevation of plasma cortisol levels has been demonstrated in both chronic and acute stress (Barcellos *et al*., 1999). The level of acute stress is widely assessed in fish by measuring the level of cortisol in plasma (Aerts *et al*., 2015). During stressful times, the levels of cortisol increases and induces an increase in glucose levels to provide energy to combat the effects of the stressor (Haiyun *et al*., 2017). Cortisol is positively correlated with glycogenolysis and gluconeogenesis in fish and negatively corelated with lipid distribution (Montero *et al.,* 1999).

Cortisol also 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 promotes 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).

High cortisol levels decrease the body's insulin output. Insulin aids in bringing sugar from the circulation into cells, where it is utilized as fuel implying that high cortisol levels decrease uptake of glucose into the cell where it plays a major role in the bioenergetics in stressed fish. Insulin increases the expression of fish glucose transporters (gluts) in liver and muscle (Polakof *et al*., 2012). Acute and chronic stress is typically associated with increased metabolic rate with plasma glucose levels being positively correlated with metabolic rate (Wendelear-Bonga, 1997). The action of cortisol is mediated through the glucocorticoid receptor (GR) which is a cytosolic receptor. Cortisol regulates the expression of genes involved in growth, metabolism and immune function (Faught

and Vijayan, 2016; Balasch and Tort 2019). Cortisol has been shown to elicit a dose-dependent reduction in specific growth rate (SGR) after 14 days in Atlantic salmon (*Salmo salar)* exposed to saline water (salinity stress) (Breves *et al*., 2020). This study also demonstrated a reduction in the levels of hepatic insulin like growth factor 1 mRNA while hepatic insulin like growth factor 1 binding protein 1(igfbp) 1b1 and -1b2 were stimulated by the high cortisol dose. Most metabolic adjustments happen in the liver and the liver has therefore been a target organ for use during stress action studies (Aluru and Vijayan 2009). Generally Cortisol upregulates pathways involved in energy substrate mobilization, including gluconeogenesis, while downregulating energy demanding pathways, including growth and immune functions.

Under stress, growth is arrested and the reproductive processes are suppressed or depressed. According to Dhabhar, (2009) acute stress has short term benefit of enhancing innate immunity unlike chronic stressors which induces immune suppression increasing the chance of infection (Tort, 2011). During a stressful event, a signaling cascade in the CNS involving the hypothalamus is triggered leading to production of catecholamines, corticosteroids- releasing hormone, adrenocortical steroid hormone, adrenal corticotropic hormone and glucocorticoids. These are the neuroendocrine mediators for the activation of the stress response. To avail the much needed energy to the stress response, the body system slows down the “less important” functions such as the immune function (Nardocci, *et al.,* 2014).

2.7 Blood Cortisol Concentrations in stressed fish

When subjected to stress, fish increases the release of cortisol from the interrenal cells via the stimulation of the SNS and CNS (Fig 2). Blood cortisol levels change with stress and indicate the state of the culture environment. Cortisol concentrations reflect the fish's stress levels. Values greater than the basal mean are indicative of a stressful environment (Montero *et al*., 1999). Different species have different basal and stressful cortisol levels, and within-species variations are influenced by factors like temperature, gender, sexual maturity, and heredity. (Martinez and Prchaz, 2009). Studies in juvenile rainbow trout

indicated that whether the fish were housed in unmixed or mixed settings, chronic plasma cortisol increase had a substantial unfavorable impact on each individual's appetite, growth rate, condition factor, and food conversion efficiency (Gregory and Wood, 1999). In brown trout mortality from common bacterial and fungal infections increases in a dose-dependent manner because of prolonged elevations in plasma cortisol levels (Pickering and Potinger, 1989). Tahir *et al.*, (2018) showed that juvenile *Epinephelus fuscoguttatus* displayed increased serum cortisol levels as compared to the controls when subjected to salinity stress. In sea bream stocking density has been shown to increase plasma cortisol levels (Ortuno *et al*., 2001). Chemical exposure in rainbow trout (Benguira *et al*., 2002) and sea lice challenge in Atlantic salmon (Bowers *et al*., 2000) have also been shown to increase the blood cortisol concentrations.

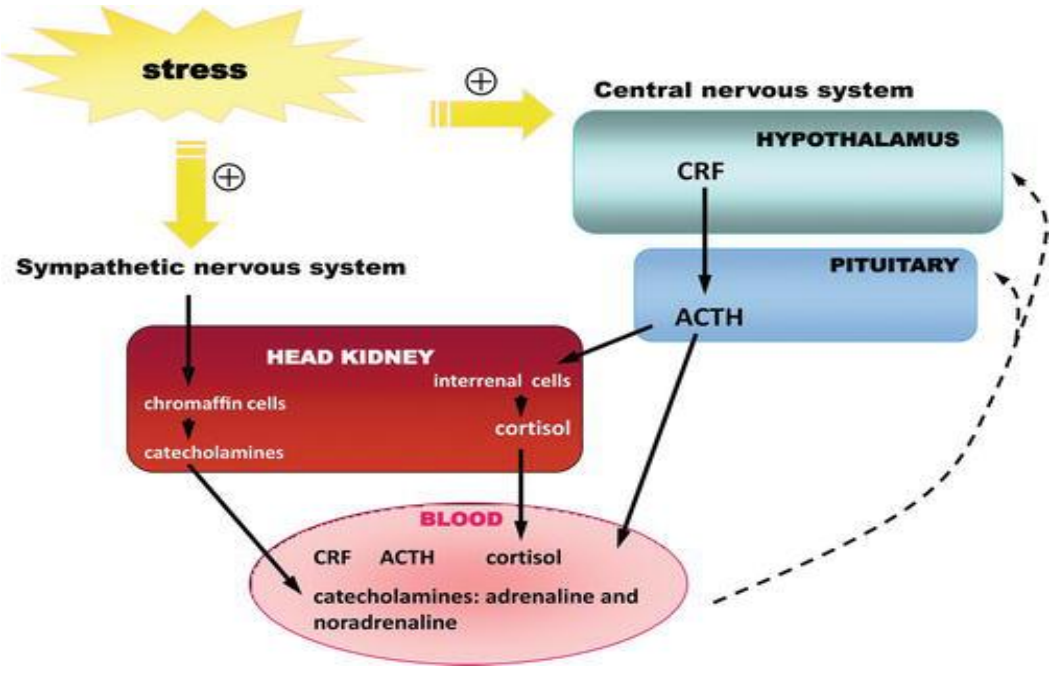

Figure 2: The cortisol release cycle. Adopted from Al-kaf, A. G. (Ed.). (2018). Corticosteroids. InTech. doi: 10.5772/intechopen.68270

2.8 Scale Cortisol Levels in fish

Plasma cortisol has for long been used as the gold standard for measurement of stress. However, the levels of chronic stress are poorly correlated with plasma cortisol levels and thus the use of fins and scale has proved to be a better indicator (Sadoul and Geffroy, 2019, Laberge *et al.*, 2019). Cortisol accumulates in scales during the growth of fish (Carbajal *et al*., 2018; Aerts *et al*., 2015) and thus can be used to extract cortisol that may act as an indicator of chronic stress in fish. Scale cortisol levels are more stable compared to plasma levels. The accumulation and clearance of scale cortisol is influenced by circulating cortisol in the blood. Change in cortisol levels in scales will occur when there is a marked and sustained variation in the circulating cortisol levels. Laberge *et al*. (2019) demonstrated that scales can provide an integrated measure of cortisol production and thus serve as an indicator of chronic stress in fish. Unlike plasma cortisol levels, scale cortisol is not affected by short time stress like handling stress that is common in aquaculture. Scale cortisol determination is taunted to be a more reliable indicator of chronic stress due to its relative stability. Furthermore, plasma cortisol levels reveal acute changes at a single point in time (Lee *at al*., 2015) and cannot tell the stress history of the fish. Blood sampling is an invasive procedure and depending on how it is carried out can in itself be a source of stress (Bertotto *et al.*, 2009) hence the need to determine cortisol levels by use of less invasive matrices.

2.9 Blood Glucose Concentration in Fish

Blood glucose level is another major signal of stress in fish. Increase in plasma cortisol as witnessed during stress induces hyperglycemic conditions in fish (Odhiambo *et al*., 2020). In hyperglycemic states, insulin is inactivated, which prevents glucose from entering cells

and elevating blood sugar levels by inhibiting the creation of glycogen. Hyperglycemia increases when fish grow in size. In addition to fish size, environmental conditions and ecosystem quality have an influence on hyperglycemia. It has been shown that there exists a positive correlation between blood glucose concentrations and increasing stress levels (Macek *et al*., 2018; Makaras *et al*., 2020). In Red spotted grouper subjected to thermal stress, Cho *et al* (2015) showed a significant increase in blood glucose concentrations during the experimental period. Stocking density stress has also been shown to increase blood glucose concentrations in juvenile Nile tilapia (Odhiambo *et al*., 2020). Research has demonstrated that there are sex variations in blood glucose levels for several teleost fish species (Eames *et al*., 2010).

2.10 Stress and Protein Metabolism in Fish

Stress results in an increase in the production of cortisol as the main glucocorticoid steroid. The action of glucocorticoids (GC) is initiated by their binding to glucocorticoid receptors (GR). Under stressful conditions, these receptors are expressed to a higher concentration especially on the skeletal muscles. Since GCs regulates protein metabolism promoting catabolism, sustained high levels of GCs may lead to muscle atrophy emanating from decreased protein synthesis and increased protein catabolism. GCs are crucial for maintaining glucose homeostasis, which occurs at the expense of Lipids and proteins. (Grammer *et al*., 2015). The degradation of proteins avails free amino acids and promotes gluconeogenesis (Bodine and Furlow, 2015). Stress therefore compromises growth performance in fish since excessive proteolysis brings about muscle atrophy.

2.11 Gene Expression in Stressed Fish

Changes in gene expression in response to various stressors such as cold (Hu, *et al*., 2016), alkalinity (Zhao *et al*., 2015), salinity (Xu *et al*., 2015), hypoxia (Li *et al*., 2017; Wang *et al*., 2017) and thermal stress (Guy *et al*., 2018; Yue *et al*., 2018) have previously been reported in fish. Stressed fish have high cortisol and circulating glucose levels in the blood (Galt *et al.,* 2018). Breves *et al.* (2020) demonstrated a dose dependent reduction in the levels of hepatic insulin like growth factor 1 mRNA with increasing concentration of cortisol in the blood of stressed fish. High cortisol has also been shown to increase hepatic insulin like growth factor 1 binding protein 1(*igfbp*) 1b1 and -1b2. Exposure of Rainbow trout to municipal waste water effluent for 14 days was shown to elicit an increase in steroidogenic acute regulatory (*StAR*) mRNA abundance, liver GR protein at the same time lowering liver hexokinase and glucokinase activities, without affecting glycogen content or the activities of phosphoenol pyruvate (PEP) carboxykinase, pyruvate kinase, lactate dehydrogenase, aspartate aminotransferase and alanine aminotransferase (Ings, *et al.,* 2011). Wang *et al*. (2008) demonstrated a modulation of the expression of Suppressor of Cytokines SOCS (SOCS-1 and SOCS-2) genes in rainbow trout subjected to stress and associated it with stress-mediated immune modulation.

According to Wiseman *et al*. (2007), rainbow trout exhibit up-regulation of 40 genes' expression after experiencing acute stress. The heightened activity of gene expression was also seen to decrease with time i.e.. 40 genes 1 hour and 15 genes at 24 hours post exposure to an acute stressor. Crowding in Nile tilapia has been shown to increase the expression of somatostatin -1 (sst1), V-fos FBJ murine osteosarcoma viral oncogene homolog Ab (fosab) and prolactin genes (Rodriguez-Barreto *et al*., 2019). There is however considerable

variation in how fish respond to a stressor because of genetic differences among different taxa and also within stocks and species (Schreck and Tort 2016). Growth hormone (*gh*), insulin-like growth factors (*igf 1* and *2*) and somatolactin (*smtla*) have been shown to influence growth performance in tilapia (Herkenhoff *et al.*, 2020). This study showed that expression of these genes were positively correlated to growth while expression of myostatin (mystn) was negatively correlated. Mystn inhibits myoblast differentiation via Smad 3 and negatively regulates myogenesis by controlling myoblasts proliferation (Hu *et al*., 2013; Langley *et al*., 2002).

Catfish with mutant Mystn gene shows both hyperplasia and hypertrophy as well as increased rate of body mass and body length gain (Khalil *et al*., 2017). Low Myostatin expression has been associated with the double-muscling phenotype while over expression of the same is linked to muscle wasting (Lee *et al*., 2010, Fuentes *et al.*, 2013). In domestic animals like cattle, mystn has been considered as an important candidate gene for studying productivity, growth and development. The dysfunction of mystn has been sighted as one of the strategies for increasing animal growth performance in livestock production (Hu *et al*., 2013). Sternstein *et al.* (2014) identified a Single Nucleotide polymorphism (SNP) in the Rabbit mystn gene which had a direct linkage with carcass composition traits. Knock out of mystn gene in rabbit has been shown to produce a rabbit breed with a double muscled phenotype (Lv *et al.*, 2016).

In some fish species e.g. brook trout and Atlantic salmon mystn production has been shown to increase in response to crowding. This increase also corresponds to an increase in circulating levels of glucose (Galt *et al*., 2018). Myogenin (Myog) is another gene whose expression signals muscle wasting. Ex-vivo experiments have showed that Mystn-1 in fish

skeletal muscle, signals glucocorticoid-induced muscle wasting via negative regulation of genes involved in muscle growth, such as *mtor, myod-2* and *myogenin*, and the induction of atrophy genes like *foxo3b* and *murf1* (Torres-Velarde *et al.*, 2018; Baehr *et al*., 2010). Among the genes involved in myogenesis, insulin growth factor gene complex Igf 1 and 2 and Myog have been singled out as reliable indicators for following muscle development and growth (Aidos *et al.*, 2020).

Overcrowding in fish may result in stress response that leads to an increase in circulating glucose. Glucose membrane transporters (GLUT) are essential proteins that help in the uptake of glucose. There are various isoforms of GLUT proteins depending on their function like GLUT 1 is involved in the transport of glucose in and out of the red blood cells while GLUT 4 performs the same function in the muscles. Aketch *et al.* (2014) demonstrated an increase in GLUT 1 expression in Tilapia (*O. niloticus*) reared at high densities. GLUT 1 is widely distributed in Tilapia and its expression has been shown to be stress induced (Hrytsenko *et al*., 2010; Wertheimer *et al*., 1991). GLUT 4 on the other hand is the major glucose transporter found in skeletal muscles. The expression of GLUT 4 has been demonstrated in fish skeletal muscle and its expression shown to increase with progression of myogenic differentiation (Diaz *et al*., 2022; Capilla *et al*., 2004). Myogenic differentiation has been shown to be associated with an increase in insulin-stimulated glucose transport.

The expression of these genes is beneficial to the fish in that it helps it escape or adopt to the stressful environmental conditions. However the expression of particular genes is limited to some fish species and not others. This could be the reason why some fish are able to tolerate particular stressors while at the same time being vulnerable to others. For

instance, Stenohaline fishes with restricted salinity tolerance ranges occupy environments with consistent salinity such as Mediterranean Moray Eel while euryhaline fishes with broad salinity tolerance ranges live in environments with changing salinity such as the Mozambican tilapia (Klutz, 2015). This intolerance may be associated with the absence or limited expression of salinity tolerance genes.

Hybridization of fish species expressing different beneficial phenotypes can result in the offspring acquiring these traits. For instance the "Sukamandi" a fast growing and salinity tolerant tilapia which was developed from the hybridization of the salinity tolerant blue tilapia with the fast growing Nile tilapia (Yu *et al*., 2022). These beneficial phenotypes are as a result of the presence of specific genes in these fish species. For instance, Dodson *et al*. (2015) showed that the North East American Rainbow Trout is a hybrid of two rainbow trout glacial races made up of differing mixtures of the two linages. These genetic hybrids showed multiple traits in excess of the values of their parental strains (Dodson *et al*., 2015). The resulting offspring bears genes from both parents and therefore exhibits genetic admixture. In addition to heterosis (hybrid vigor) at the individual level and/or substantial genetic variation for pertinent phenotypic characteristics at the population level, hybridization may also have beneficial impacts on fitness. These two characteristics could enable hybrids to adapt to new and/or different environments more successfully than their parental lineages. Such hybridizations leading to introgressions form the basis of breeding programs aimed at improving fish strain performance.

Various methods have been used to determine genetic sequence with the single reference analysis method being the most prominently used. However, the single reference analysis method has suffered shortcomings in the identification of genetic variations among

populations. These shortcomings, however, can be effectively overcome by constructing a pangenome. A pangenome aims to capture the complete genetic diversity within a species and reduce bias in genetic analysis inherent in using the single reference genome (Hurgobin and Edwards, 2017). The complete set of genes in a particular species is known as the pangenome. It is made up of the essential genes, which are found in every member of the species, and the variable, auxiliary, and disposable genes, which are found in some but not all members of the species.

According to the assessment of the pan-genome, the species either possesses an open or closed pan-genome. It is either a closed pan-genome, where more sequenced genomes do not add new genes to the current pan-genome, or an open pan-genome, where the number of genes in the pan-genome rises with the addition of other genomes. In addition to having an open pan-genome, species that colonize various settings are able to interchange genetic material with ease. While species living in isolated habitats have closed pan-genomes and fewer opportunities to exchange genetic material. Consequently, pan-genome analysis provides a framework for identifying and comprehending genomic diversity. Using pangenomes for phylogenetic analysis has the benefit of allowing SNPs found in PAV-displaying areas to be utilized to infer more precise connections between accessions. The phylogenetic tree may be used to plot the numbers that represent which genes are specifically present and absent in each accession using variable genes. The amount of nucleotide changes per site determines the length of each branch in the tree, and the evolutionary patterns shown in the tree may be connected to the biological characteristics of each accession.

Pangenome analysis is mostly useful in the identification of Single nucleotide Polymorphism Markers (SNPs) which aid in the identification of the genetic variance within a species. SNPs are high-resolution molecular markers, used to analyse the neutral and adaptive genetic diversity of populations with large numbers (Wenne, 2023). SNPs calling has been used to identify species and hybrids in natural environments, as well in examining the genetic implications of restocking as a conservation effort and the deleterious consequences on wild populations of fish accidentally escaping from culture systems (Williams *et al.,* 2010). SNPs are extremely beneficial for identifying genomic regions associated with phenotypic polymorphisms that are important for aquatic biodiversity conservation and management. Traditionally, species evolution has been understood as a long-time process, lasting up to hundreds or thousands of years (Wiens, 2004). Nonetheless, there is rising evidence that recent speciation occurs in natural environments resulting in morphological divergence. Recent improvements in sequencing techniques have led in the collection of massive data sets of molecular markers useful in identifying genetic diversity in populations and genomic areas influenced by natural selection (Bansal and Boucher, 2019). SNP loci can be found in coding or noncoding genome regions and are useful markers in defining populations and species that are closely related, characterisation of their polymorphism and how it changes over time and helps in the understanding of the adaptations and genetic polymorphism, of a population, at specific trait loci (Garg *et al.,* 1999). The current study sought to identify and quantify instances of stress in cultured Nile tilapia (*O. niloticus*). In order to shed further light on the adaptive and genetic mechanisms involved in response to chronic stress, the study attempted to

identify probable genes and biological pathways related with the response to chronic stress in Nile tilapia (*O. niloticus*).

SNPs were used in the current study to determine the links between aquatic animal populations in their natural habitats. The discovery and development of Nile tilapia strains that are highly productive and resilient to environmental stress would benefit from this study. Additionally, this study sought to identify genes that would enable fish to produce highly under conditions of prolonged stress.

CHAPTER THREE

MATERIALS AND METHODS

3.0 Materials

Hand sexed male Nile tilapia (*O. niloticus*) mean weight 25 ±1.25 g; total length 8 ± 0.35 cm were obtained from Ilala hatchery in Shinyalu sub county of Kakamega County, Kenya, and reared in the laboratory for 2 weeks prior to the start of the experiment. During the acclimatization and experimental period, the fish were maintained on a 12 hr. light and 12 hr. dark photoperiod cycle. Feeding was done two times a day with commercial feed 2 mm diameter pellet size containing 32% of gross protein and 3,500 Kcal/kg of digestible energy. The fish were fed to satiation for 30 minutes each time any feed remaining uneaten after 30 minutes was siphoned out and discarded. The use of only male fish was to reduce unwanted variations that may result from sex differences (Zabegalove *et al.,* 2019). Spring water from Lutonyi fish farm where the experimental set-up was hosted was used for rearing the fish. The fish were reared from November 2021 to March 2022.

3.1 Experimental Design

3.1.1 Fish Ammonia Stress Experiment

A total of 525 healthy tilapia juveniles (25 ± 1.5 g) were randomly divided into seven groups and stocked in 21 white circular 500 L polyethylene tanks in a static system aerated by blowers connected to air stones. Three replicates of 25 fish per tank as guided by Thorarensen, *et al.* (2015) were used. The tanks were fitted with calibrated thermostat set at 26 ^{o}C for temperature control. The dissolved oxygen concentration and temperature were measured with a digital oximeter (Hydrolab MSIP-REM-HAH-QUANTA (USA). The dissolved oxygen was always maintained at levels above 6mg/L while the temperature was

maintained at 26 ± 1 ^{o}C for the period of the experiment. The pH was measured using a digital pH meter and maintained at 6.8± 0.2. The treatments were distributed as follows:

The first (control) group were maintained in spring water without addition of ammonia throughout the growth period.

Six groups of 25 fish per tank were maintained at different concentrations of un-ionized ammonia (0.4, 0.8, 1.2, 1.6 and 2.0 and 2.4 mg/L) throughout the growth period.

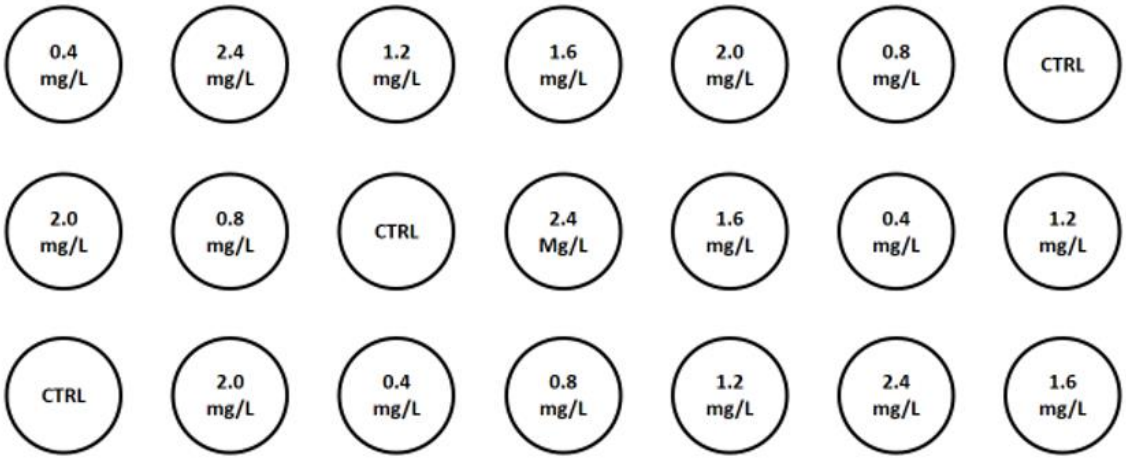

Figure 3: experimental layout for the ammonia treatment

Each of the treatments above were replicated twice with the treatments being randomly assigned within the blocks (Fig 3). Each tank was filled with the right amount of ammonium chloride, which was then dissolved to create the various ammonia concentrations. The unionized ammonia concentration was calculated using the formula developed by Emerson *et al*. (1975)

F = 1/(10(pKa-pH)+ 1) where **pKa = 0.0901821 + 2729.92/Tk**

- Where: Tk is temperature in degrees Kelvin
- F is the proportion of the un-ionised ammonia

The fish were then reared for 70 days and their growth monitored every fortnight. Growth was monitored by measuring the total length, weight and determining the condition factor, growth rate and specific growth rate (SGR) of the fish.

3.1.2. Fish Stoc king Density Experiment

A total of 1,185 Nile tilapia (*O. niloticus*) juveniles were randomly distributed in 21 white circular polyethylene tanks (500 L). Each tank was assigned a treatment as follows:

The control group were stocked at 20 fish per tank. Six groups of fish were maintained at different stocking densities (25, 40, 55, 70, 85 and 100 fish per tank).

Each of the treatments above were replicated twice. Each of the treatments were randomly assigned within the blocks (Fig 4). 20% water exchange was carried out every day to allow for the siphoning of the left-over feeds and the excrement in each tank.

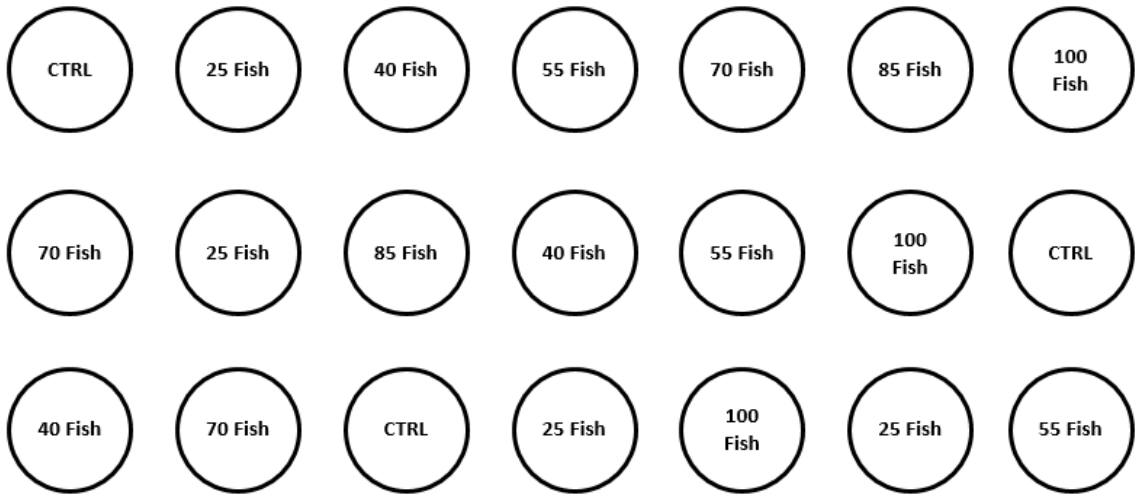

Figure 4: Experimental set-up for the stocking density treatment.

The fish were subsequently raised for 70 days, and every two weeks their growth was monitored by measuring the fish's total length and weight then calculating the condition factor, growth rate, and specific growth rate (SGR) of the fish.

3.2 Blood and scale samples Collection

Three fish from each treatment were randomly sampled. The selected fish were anaesthetised in 3-aminobenzoic acid ethyl ester methane-sulfonate (MS-222, Sigma, USA) for physiological parameter measurements. Blood and scale were removal for cortisol levels determination. The fish were then sacrificed and 2 g of muscle in the dorsal caudal region sampled.

3.3 Sample Processing

3.3.1 Physiological Parameters Measurements

The total length and final live weight of the fish were determined and used to calculate the condition factor, growth rate and specific growth rate of fish in every treatment.

3.3.2 Calculation of growth Performance

Specific growth Rate of the fish were determined by the formula:

SGR (%) = (Ln final weight of fish – Ln initial weight of fish) /duration of experiment) * 100

Condition factor K (CF %) = (body weight /total length) *100

3.3.3 Determination of Cortisol Levels

Two millilitres of blood were collected from each sampled fish by venepuncturing the tail vein using disposable K_2EDTA (0.5 M, pH 8.0) treated syringe and a gauge 30 needle. The blood was maintained on ice throughout the sampling process and spun down (3000 g at 4 ^{o}C for 10 minutes). The plasma was removed and transferred to a capped polypropylene tube and frozen at −80°C until analysed.

The quantification of Cortisol was done using competitive inhibition enzyme linked immunosorbent assay (ELISA) using the CUSABIO® fish cortisol ELISA kit following the manufacturers instruction. The plates used were pre-coated with a standard cortisol antigen. The competitive inhibition reaction was launched between pre-coated cortisol antigen and cortisol in the samples. Where 50 µl of either the sample or cortisol standards were aliquoted to each well in duplicates with two blank wells that were not filled with either the standard or sample solution. 50 µl of antibody was added to each well except the blank well and mixed thoroughly by shaking the well for 60 seconds. The plate was then covered with an adhesive strip and incubated at 37 ^{0}C for 40 minutes. The plate was then washed and left to stand for 2 minutes. 100 µl HRP- conjugate were added to each well (except to the blank well) and covered with an adhesive strip and incubated for 30 minutes at 37 ^{0}C. The plate was then incubated for 2 hrs. at room temperature and washed five times in wash buffer before adding 90 µl of substrate solution (3,3',5,5'-tetramethylbenzidine (TMB) to each well and incubating at 37 ^{0}C for 20 minutes in the dark. The reaction was stopped by adding 100 µl of stop solution (0.5 M sulphuric acid) and absorbance measured immediately at 450 nm and 540 nm. The concentration of the cortisol was obtained from a standard curve constructed from readings obtained from the same run.

3.3.4 Determination Scale Cortisol levels

Measurement of scale cortisol levels was used to estimate the stress levels in the experimental fish. After anaesthetizing the fish ten scales were removed from along the lateral line using a clean pair of forceps. The scales were washed thrice with 3 ml of isopropanol for 2.5 minutes for each wash and air dried on a tissue paper at room temperature. The dried scales were then chopped into fine particles using a clean pair of

scissors. The cortisol was extracted by incubating 0.100 ± 0.01 g of the fine particles in 8 ml of methanol for 16 hrs. at 35 ^{0}C. The mixture was then centrifuged at 3000 x g for 10 minutes and the supernatant decanted into a fresh tube and the solvent evaporated under a stream of nitrogen at 38 ^{0}C until dry.

The resting powder was dissolved in 200 µl of sample diluent and used to determine the cortisol concentration as described in 3.3.3 above.

3.5 Gene Expression Studies

3.5.1 RNA Extraction

Muscle tissues sampling was done by making an incision on the left side of the fish in the region between the lateral line, the dorsal and caudal fins. The tissue was quickly chopped into small pieces approximately 0.5 cm wide and quickly ground in liquid nitrogen.

The tissues were homogenized in TRIzol™ (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instruction. The ratio of TRIzol to tissue used 1 ml TRIzol for 70 mg of tissue. To ensure the integrity of the RNA all the materials used were treated with RNAse Away (Invitrogen, Carlsbad CA, USA).The cells were lysed by adding chloroform and the mixture was incubated for 3 minutes then centrifuged at 12,000 x g for 15 minutes at 4 ^{0}C. The upper aqueous phase was transferred to a new tube and RNA precipitated by addition of isopropanol. The RNA was pelleted by centrifuging at 12,000 x g for 10 minutes at 4 ^{0}C. The pellet was resuspended in 75% ethanol then centrifuged at 7500 × g at 4 ^{0}C for 5 minutes. The resulting pellet was air dried and resuspended in RNase free water.

The RNA quality was assessed using the Nanodrop-2000 (Thermo Fisher Scientific, United States) and electrophoresis in 1.5% agarose gel. The concentration of each of the samples

was then adjusted to 500 ng μl^{-1}. DNAse I (Invitrogen) was added to each sample to eliminate any genomic DNA present. Three samples from each treatment were combined together in equal proportions prior to the library construction.

3.5.2 RNA Library Construction

Using poly-T Oligo-attached magnetic beads, messenger RNA was isolated from total RNA. After fragmentation, random hexamer primers were used to synthesize the first strand of cDNA, and either dUPT or a directed library were used to synthesize the second strand. End repair, A-tailing, adaptor ligation, size selection, USER enzyme digestion, amplification, and purification were all completed before the library was ready. Qubit, real-time PCR, and a bioanalyzer were used to assess the library's accuracy in quantification and size distribution detection. The Libraries were quantified and sequenced on Illumina platform in accordance with the efficiency of library, concentration and the volume of data.

3.5.4 RNA Sequencing

The mRNA and tRNA were separated using an oligo (dT) magnetic bead from Invitrogen USA. The acquired mRNA was then broken up into small pieces with a 300 bp size limit using a fragmentation buffer. DNA Polymerase I and RNAse H were utilized in the synthesis of the second strand cDNA, which was then followed by adapter ligation and 15 cycles of PCR amplification. The final cDNA library was created by separating the PCR product on 2% agarose gels. TBS-380 pcogreen (Invitrogen) was used to quantify the libraries before being sequenced using the Illumina Hiseq 4000 platform (majorbio Biotech Co., Ltd., Shanghai, China) to produce 2 x 150 BP end reads.

3.6 Reads Mapping of the Experimental Sample to the *O. niloticus* Reference Genome

Reference genome and gene model annotation files were downloaded from genome website directly. Hisat2v2.0.5 was used to build the index of the reference genome (Pinero *et al*., 2021). Paired-end clean reads were then aligned to the reference genome using Hisat2 v2.0.5. Hisat2 was chosen as the mapping tool because, in comparison to other non-splice mapping methods, it can create a database of splice junctions based on the gene model annotation file.

3.7 Quantification of Gene Expression Levels in the Experimental Sample

The reads mapped to each gene were counted using FeatureCountsv1.5.0-p3 (Liao *et al*., 2013), and the expected number of Fragments Per Kilo base of transcript sequence per Millions base pairs sequenced (FPKM) of each gene was then determined based on the length of the gene and the reads mapped to it.

3.8 Differential Gene Expression Analysis

Differential expression analysis of two conditions/groups (two biological replicates per condition) was performed using the DESeq2R package (1.20.0) (Reshef *et al*., 2017). DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate (Sarkar and Tang, 2021). Genes with an adjusted P-value ≤ 0.05 found by DESeq2 were assigned as differentially expressed for edge R without biological replicates. Prior to differential gene expression analysis, for each sequenced library, the read counts were adjusted by edge R program package through one scaling normalized factor. Differential expression analysis of two conditions was performed using the edge R

package (3.22.5). The P-values were adjusted using the Benjamini and Hochberg method. Corrected P-value of 0.05 and absolute fold change of 2 were set as the threshold for significantly differential expression.

3.9 Enrichment Analysis of Differentially Expressed Genes

Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the Cluster Profiler R package (Kim *et al*., 2015), in which gene length bias was corrected. GO terms with corrected P-value less than 0.05 were considered significantly enriched by differential expressed genes. Cluster Profiler R package was used to test the statistical enrichment of differential expression genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Disease Ontology (DO) pathways with corrected P-value less than 0.05 were considered significantly enriched by differential expressed genes. The DisGeNET database integrates human disease-related genes. DisGeNET pathways with corrected P-value less than 0.05 were considered significantly enriched by differential expressed genes. Cluster profiler software was used to test the statistical enrichment of differentially expressed genes in the Reactome pathway, the DO pathway, and the DisGeNET pathway.

3.10 Real Time qPCR

To verify the reliability of the RNA-Seq results obtained, RT q-PCR was performed on four selected genes. From the two treatments, one DEG from each of the up and down regulated DEGs was randomly chosen. Four DEGs (DUSP-1 and Mych (down-regulated), Pfkma, and Acetyl CoA Thioesterase 4 (up regulated)) were the randomly selected DEGs for RT-qPCR. RNA was extracted using TRIzol reagent (Invitrogen, Washington, Waltham, MA, USA). This was used to prepare a cDNA library using RevertAid First

strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The reaction mixture consisted of 10 µg template, 2 µL dNTPs (Thermo Fisher Scientific, Waltham, MA, USA), 2 µL of 10 x Dream Taq buffer (Thermo Fisher Scientific, Waltham, MA, USA), 0.25 µL of 5U/µL Dream Taq DNA, 20 x Evagreen®Dye (BIOZOL Diagnostica Vertrieb GmbH, Eching, Germany) (Wekesa *et al*., 2022), 1 µL of 10 pM forward and reverse primers and topped up to 20 µL with water. The primers used (Table 1) were designed using primer3plus (Untergasser *et al*., 2007). Real time qPCR was performed on CFX Connect Real-Time PCR optical detection System (Bio-Rad, Hercules, CA, USA). The qPCR amplification products of the four genes were normalized with those of glyceraldehyde-3-phoshate dehydrogenase gene.

Table 1: Primers sequences for selected genes used during the RT-qPCR validation of the RNA-seq results.

Gene name	Primer sequence	Annealing temp. (°C)	Amplicon size (bp)	GC content (%)
dusp1_F	ACTTGAACACATCGTCCCCAAC	61.1	181	50.0
dusp1_R	TGTGCCGTCCTTTTTCACTTGG	61.8		50.0
mych_F	AACTGTTTCATGCGCTTGCG	60.7	190	50.0
mych_R	TTTCCTGGTTTGCAGTCTGTGG	61.3		50.0
pfkma_F	TGCAATTAAGGCCAAGCACCAC	62.2	196	50.0
pfkma_R	GCATGGTGTTGTAAAGGCTCACC	62.5		52.2
AcoT4_F	TTGAGGCAGGTGGTGAAGACAAG	62.9	185	52.2
AcoT4_R	TGTGCAACATAACAGGCAGCTG	62.0		50.0
GAPDH_F	TGGCATTGCACTCAACGACAAC	62.5	182	50.0
GAPDH_R	GTGCAGCAAACAAGCTTTGGTC	61.6		50.0

3.11. Sample Collection for phylogenomics study

Female Nile tilapia was collected from a local pond in Ilala Fish farm in Kakamega East Sub-County, Kakamega County, western Kenya. Ilala fish farm is one of the hatcheries providing fingerlings to farmers in Kakamega County and western Kenya in general. Genomic DNA from muscle tissue was extracted using Qiagen GenomicTip100 (Qiagen, Germantown, MD, USA).

3.12. Fish Muscle DNA Extraction and Sequencing

DNA was extracted from the dorsal caudal muscle tissue of the selected fish using the protocol described in Mayjonade *et al*. (2016). Tissue samples of about 50 mg were ground to a fine powder in liquid nitrogen. The powder was then placed in 1.5 mL microtubes containing 0.7 mL 2% CTAB extraction buffer [20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 2% CTAB, plus 0.4% β-mercaptoethanol added just before use]. The solution was incubated at 65ºC for 45 min, gently mixing by inversion every 15 minutes; 500 mL of chloroform-isoamyl alcohol (24:1) was added to the tubes and gently mixed for 1 minute. Samples were centrifuged for 10 minutes. at 12,000 rpm; 0.6 mL of the supernatant was then transferred to a fresh tube following the addition of 500 µL chloroform-isoamyl alcohol (24:1); this procedure was repeated twice. 500 µL of the supernatant was then transferred to a fresh tube with 0.7 mL of cold isopropanol (-20ºC). Samples were gently mixed by inversion and centrifuged at 12,000 rpm for 10 min. This allowed for visualization of the DNA adhered to the bottom of the tube. The liquid solution was then released and the DNA pellet washed with 1 mL of 70% ethanol to eliminate salt residues adhered to the DNA, and set to dry for approximately 12 h, or until the next day, with the tubes inverted over a filter paper, at room temperature. The pellet was then re-suspended

in 100 mL TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) plus 5 mL Ribonuclease (RNAse 10 mg mL–1) in each tube; this solution was incubated at 37ºC for 1h, and after stored at -20ºC. DNA quality and fragment lengths were checked using the DNA screentape (Tape station analysis software a.02.01 sri). The isolated genomic DNA was subsequently applied to construct DNA sequencing libraries using illumina MiseQ. The raw reads were further trimmed by removing 5 bases in both ends. Duplicated reads and reads with 10 or more Ns and low-quality bases were discarded to improve the quality of sequenced reads.

3.13. Genome Assembly and Annotation

Genome was assembled de-novo by Abbys v2.8.5 and gaps filled with ABYss to construct contigs and original scaffolds by using clean reads. The genome completeness was checked by BUSCO v4.1.2 and contigs less than 500 were removed by inhouse bash script. Raw reads and the genome assembly have been deposited in the National Centre for Biotechnology Information (NCBI) under the project ID PRJNA848236. Repeat sequences in the F1 Isolate assembly were predicted by an integration of three routine approaches, including de novo, tandem repeat predictions and homology annotation (Mitra *et al*., 2021). For the de novo prediction, RepeatMasker v4.0.9_p2 was used to identify and mask the repeats (Tarailo-Graovac and Chen 2009). Ab initio gene prediction was performed using Augustus v3.5.0 and GeneMark- ES. Augustus was run on a predefine *Danio rerio* genome training set. The tandem repeats were subsequently predicted using Tandem Repeat Finder (Benson 1999) (version 4.04). These repeat data from above three approaches were integrated to generate a non-redundant repeat set. For the homology annotation, protein sequences of *Danio rerio, Oreochromis niloticus, Oreochromis aureus, Betta splendens,*

Latimeria chalumnae, Siriperca chuatis and *plecoglossus altivelis* were downloaded from the Ensembl database (release 70). These sequences were aligned onto the F1 strain assembly to generate alignments using Python script with an e-value < 1.0 10.5. Python script was used to select consensus genes predicted by both Augustus v3.5.0 and GeneMark-ES.

3.14. Comparative Genomics

Orthologous analysis was performed with orthoMCL and COGtriangles programs and perlscript from GET_HOLOGUES program was used to generate pan genome matrix from intersection between orthologues generated by both programs.

Gene families were identified by OrthoMCL (v1.4). First, nucleotide and protein data of six species representative of different teleost families (*O. niloticus, B. splendens, O. aureus, D. rerio, L. chalumnae, S. chuatsi* and *P. altivelis*) were downloaded from Ensembl (Release 70) and National Center for Biotechnology Information to coanalyze with the experimental sample genome assembly (Figure29). The longest transcript of a gene was retained among the different alternative splicing transcripts, genes with ≤30 amino acids were discarded, and then an "all against all" BLASTP comparison was performed followed by filtering using "E-value ≤ 1E-07" cutoff. The blastp alignments were clustered using OrthoMCL (v1.4) (Fischer *et al*., 2011) with a 1.5 inflation index.

3.15. Construction of the Phylogenetic and Divergence Time Trees

Phylogenomic analysis was produced by GET_PHYLOMARKERS program in which implements the Figtree program. Protein sequences of each single-copy gene family were aligned to each other. The protein alignments were then converted to their corresponding coding sequences using an in-house Perl script. These nucleotide sequences were linked into a continuous sequence for each species. Non-degenerate sites, obtained from the

continuous sequence of each species, were then joined into a new sequence of each species to build a phylogenetic tree using MrBayes (Huelsenbeck and Ronquist 2001) (Version 3.2, with the GTR + gamma model). The tree was then visualised using the figtree program which revealed isolate F1 as closely related to *O. niloticus, O. aureus* and *B. splendens*. To determine the number of genes shared or unique between them, a Venn diagram was constructed using the Vennplex (Cai *et al*., 2013).

3.16 Ethical Considerations

The experiments in this research were carried out in accordance with the ethical principles in animal experimentation. The experiments were done in a controlled environment to avoid contamination of the fish rearing facilities. Approval were obtained from Masinde Muliro University of Science and Technology's Institutional Scientific Ethics Review Committee (ISERC) (Appendix 2) and subsequently research permit was obtained from National Council of Science, Technology and Innovations (NACOSTI) before commencement of this research (Appendix 3).

3.17 Statistical Analysis

Data were presented as the mean ± standard errors (Mean ± S.E.) and were then subjected to one-way ANOVA analysis of variance (IBM SPSS for Windows version 26) to determine the significance of the difference between treatments. The errors were reported at 95% confidence interval. The data were then subjected to Tukey *post hoc* test (at the 5% significance level) to determine which treatments are truly different.

CHAPTER FOUR

4.0 RESULTS

4.1 Growth Performance for *O.niloticus* Subjected to Chronic Stress

Length weight measurements were used to determine the growth performance of Nile tilapia subjected to stress. The two parameters were used to calculate the fish specific condition factor and specific growth rates (SGR). Higher ammonia concentrations in aquaculture resulted in significantly lowered SGR values compared to the controls (Table 2). The stocking density treatment also showed a similar trend as shown in Table 3. The growth rates were positively correlated with the relative condition factor (K_n) in both cases. The K_n decreased with increase in ammonia concentrations in the culture water and also decreased with increase in stocking densities (Table 2 and 3).

Table 2: Length weight relationship for Nile tilapia subjected to ammonia treatment.

			Length weight Relationship			
Ammonia Conc. (Mg/L)	**Fish Length (cm)**	**Fish Weight (g)**	**a**	**B**	**K_n**	**SGR (%)**
Control	13.4 ± 1.85	56.32 ± 22.28	0.01397	3.12202	1.17	1.81341
0.4	13.2 ±1.67	52.56 ± 20.92	0.01415	3.12788	1.13	1.78352
0.8	13.1 ±1.33	51.18 ± 17.88	0.01418	3.1277	1.11	1.64517
1.2	13.0 ±1.80	46.37 ±17.86	0.01437	3.11417	1.08	1.56446
1.6	12.8 ±1.39	40.93 ± 12.30	0.01484	3.08808	1.02	1.5114
2.0	12.7 ±1.34	38.70 ± 10.00	0.0154	3.07442	0.99	1.3249
2.4	12.5 ±1.14	35.09 ± 8.87	0.01586	3.06278	0.93	1.22908

Fortnightly mean Lengths and weights ± standard deviation (SD) of fish subjected to different ammonia concentrations. K_n represents the specific condition factor values and the SGR represents the specific growth rate values "a" is the exponent describing the rate of change of weight with length of the fish while "b" is the weight of fish at unit length.

Table 3: Length weight relationship for Nile tilapia (*O. niloticus*) subjected to stocking density treatment.

Density	Length-Weight Relationship					
Fish / Tank	**Length (cm)**	**Weight (g)**	**a**	**B**	**K_n**	**SGR (%)**
Control	14.1 ± 1.95	52.05 ± 21.34	0.012961	3.0635	1.17	1.567213
25	14.0 ± 1.86	48.54 ± 20.46	0.013067	3.0524	1.14	1.540296
40	13.8 ± 1.66	46.44 ± 17.64	0.013103	3.0601	1.11	1.329046
55	13.7 ± 1.65	41.62 ± 10.88	0.013156	3.0578	1.07	1.065474
70	13.4 ±1.19	37.10 ± 8.46	0.013323	3.0490	1.01	0.905001
85	13.1 ± 1.17	34.34 ± 8.15	0.013327	3.0711	0.95	0.893896
100	13.1 ± 1.21	32.20 ± 7.43	0.013445	3.0627	0.91	0.812368

*Fortnightly mean Lengths and weights ± standard deviation (SD) of fish subjected to different stocking densities. K_n represents the specific condition factor values and the SGR represents the specific growth rate values "a" is the exponent describing the rate of change of weight with length of the fish while "b" is the weight of fish at unit length.

To determine the impact of stress on the weight gain of Nile tilapia under chronic stress, a plot of weight against time was drawn **(Figure 5 (a) and (b)).** In the ammonia treatment it is evident that the higher the ammonia concentration, the lower the weight gain **Figure 5 (a)**. Similarly, higher stocking densities resulted in slower weight gain rates **Figure 5 (b**). At the onset of the experiment, the growth was almost at per for all the treatments. For ammonia treatment, the difference in weight gain began to be evident from the fourth week while for the fish under stocking density treatment showed the difference from the sixth week. During the period of experimentation eight fish died. The fish that died were distributed in the various tanks as follows; 2 fish in the tanks with 2.4 mg/ L ammonia, two from the 1.2 mg/L tank and one from the 1.6 mg/L tank. From the stocking density treatment, two fish died in the 100 fish per tank treatment and one died in the 55 fish per

tank experiment. The reported mortality however, did not significantly affect the outcome of the experiment

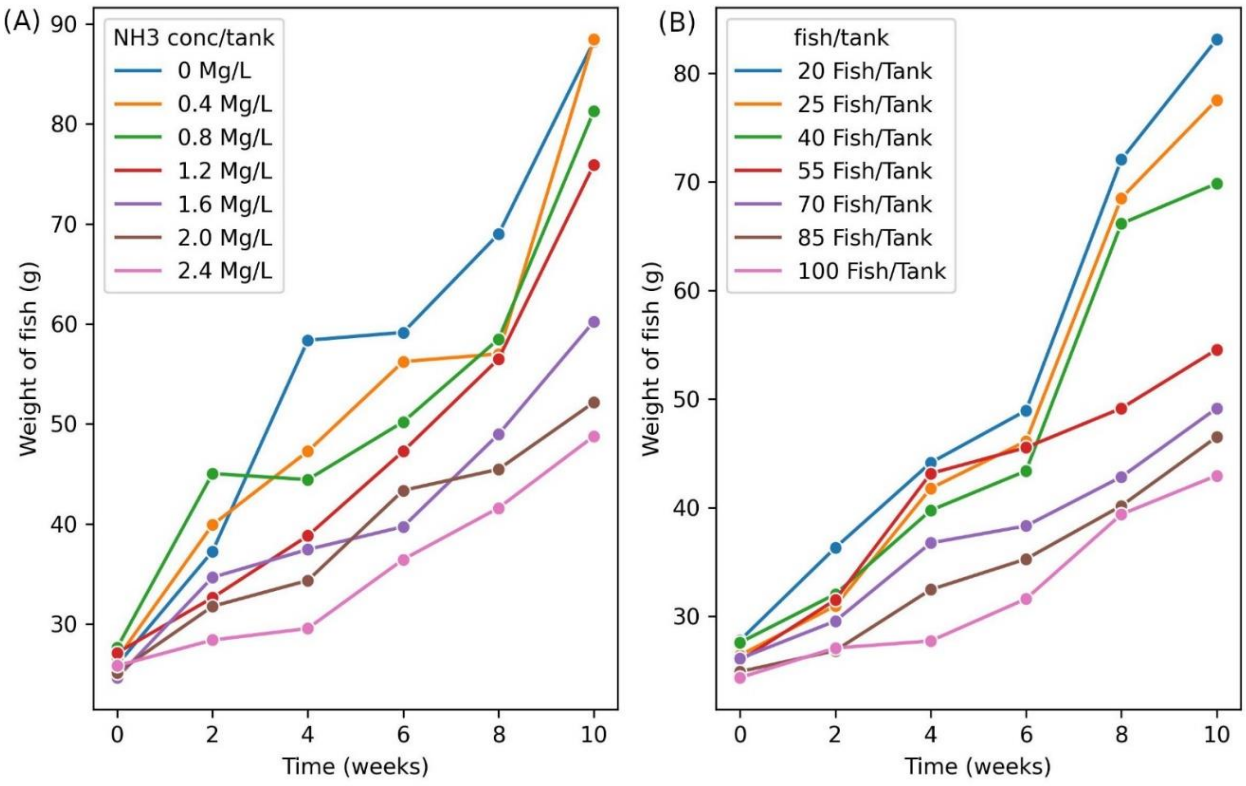

Figure 5: Fortnight weight gain for Nile tilapia subjected to chronic stress. (A) Weight gain for fish in ammonia treatment. Each line represents the average variation of weight with time at the different ammonia concentrations. (B) Weight gain for fish in stocking density treatment. Each line represents the average variation of weight with time at the different stocking densities.

4.2 Blood Glucose and Cortisol Concentration

Results of the current study indicated that the glucose concentration in plasma of experimental fish was significantly higher in ammonia treatments compared to the controls (i.e., mean± SD: 26.91±4.32 mg/dL Vs 18.23±4.1 mg/dL (Table 6). Similar observations were made for the stocking density treatment where the mean cortisol concentration was 25.71 ±3.72 ng/ml vs 22.47± 4.10 mg/dL for the control (Table 7). The plasma glucose levels increased proportionally with increase in ammonia concentrations (i.e. 18.23 ± 4.1

mg/dL to 30.88 ± 4.27 mg/dL) and stocking densities (17.47 ± 4.10 mg/dL to 30.34 ± 4.27 mg/dL) respectively (Table 6 and Table 7 respectively). Although there was a significant difference between the treatments when compared to the controls, there was no significant difference when the treatments were compared to each other.

There was a significant difference in the mean± SD plasma cortisol concentration between the controls and the ammonia treatments ($p< 0.05$) i.e. 3.65 ± 0.73 ng/ml and 5.11 ± 1.00 ng/ml respectively (Table 4). There was a significant difference in the mean± SD plasma cortisol concentration between the controls and the stocking density treatments ($p< 0.05$) i.e. 3.65 ± 0.73 ng/ml and 5.11 ± 1.00 ng/ml respectively (Table 5).

Table 4: Mean plasma cortisol concentrations in Nile tilapia after subjecting to increasing ammonia concentrations.

Ammonia Conc. (mg/L)	**Mean plasma cortisol concentration (ng/ml)**			**Std. Deviation**
CTR	4.71^{a}			0.52
0.4	5.72^{ab}	5.72^{ab}		0.82
0.8		6.09^{bc}	6.09^{bc}	0.63
1.2		6.23^{bc}	6.23^{bc}	0.84
1.6		6.68^{bc}	6.68^{bc}	1.03
2.0			7.08^{c}	0.75
2.4			7.22^{c}	0.82

*The superscripts indicates significant difference at $p<0.05$ of the mean plasma cortisol concentration following ammonia treatment. Treatment with 0.8 mg /L and above had significantly higher plasma cortisol levels compared to the control.

Table 5: The mean plasma cortisol levels of Nile tilapia after subjecting them to stocking density treatment.

Stocking density (Fish / tank)	**Mean cortisol Concentrations (ng/ml)**			**Std. Deviation**
CTR	3.65^{a}			0.73
25	4.75^{ab}	4.75^{ab}		0.50
40		4.81^{b}		0.48
55		5.09^{b}		0.66
70		5.36^{bc}	5.36^{bc}	0.99
85		5.74^{bc}	5.74^{bc}	0.47
100			6.4^{c}	0.42

The superscripts indicates significant difference at $p<0.05$ of the mean plasma cortisol concentration following stocking density treatment. Treatment with 40 fish / tank and above had significantly higher plasma cortisol levels compared to the control.

Table 6: Mean Plasma Glucose levels of Nile tilapia after subjecting them to ammonia treatment.

Ammonia Conc. (mg/ml)	**Mean plasma glucose Concentrations (mg/dL)**			**Std. Deviation**
CTR	18.23^{a}			4.10
0.4	21.73^{ab}	21.73^{ab}		2.69
0.8		23.63^{b}		2.54
1.2		26.16^{bc}	26.16^{bc}	2.23
1.6			29.01^{c}	3.39
2.0			30.07^{c}	1.52
2.4			30.88^{c}	4.27

*The superscripts indicates significant difference at $p<0.05$ of the mean plasma glucose concentration following ammonia treatment. Treatment with ammonia concentration of 0.8 mg/L and above in the culture water had significantly higher plasma glucose levels compared to the control.

Table 7: Mean Plasma Glucose Concentration for Nile tilapia after subjecting them to Stocking Density Treatment

Stocking density	Mean plasma glucose levels (mg/dL)			Std. Deviation
CTR	17.47[a]			4.10
25	23[ab]	23[ab]		2.69
40		23.27[b]		2.54
55		24.62[b]		2.23
70		25.71[bc]	25.71[c]	3.39
85		27.32[bc]	27.32[c]	1.52
100			30.34[c]	4.27

*The superscripts indicates significant difference at $p<0.05$ of the mean plasma glucose concentration following stocking density treatment. Treatment with 40 fish / tank and above had significantly higher plasma glucose levels compared to the control.

Similarly, the scale cortisol levels in the stocking density treatments showed a significant difference at ($p<0.05$). The mean cortisol concentration of all the treatments were significantly different from the controls i.e. 2.47 ± 0.04 mg/g vs 2.69 ± 0.15 mg/g (Table 9). Separation of means using Turkeys HSD, gave three homogenous subsets i.e. Low (25 fish per tank), medium (40 fish – 55fish per tank) and High stocking densities (70 fish – 100 fish per tank) with an insignificant difference ($p>0.05$) within these categories. Result of the current study indicates a positive correlation between scale and plasma cortisol.

This study showed a significant difference ($p < 0.05$) in the levels of scale cortisol when each ammonia treatment was compared to the controls i.e., 2.54 ± 0.13mg/g vs 2.80 ± 0.13 mg/g for ammonia treatment (Table 8). Separation of means by Turkey's HSD gave two homogenous subsets between the different ammonia treatments i.e., Low ammonia concentration (0 mg/ml-0.8 mg/ml) and High ammonia concentration i.e. (1.2 mg/ml-2.4 mg/ml). There was no significant difference ($p > 0.05$) in the scale cortisol content between the categories.

4.3 Scale Cortisol Concentration

Table 8: The mean scale cortisol levels after subjecting Nile tilapia (*O. niloticus*) to ammonia treatment.

Ammonia Conc. (mg/L)	**Mean scale cortisol concentrations (mg/g)**		**Std. Deviation**
CTR	2.54^{a}		0.13
0.4	2.75^{ab}	2.75^{ab}	0.08
0.8	2.76^{ab}	2.76^{ab}	0.14
1.2		2.79^{b}	0.16
1.6		2.81^{b}	0.08
2.0		2.84^{b}	0.09
2.4		2.89^{b}	0.18

*The superscripts indicates significant difference at $p < 0.05$ of the mean scale cortisol concentration following ammonia treatment. Treatment with concentrations of 1.2 mg/L ammonia in culture water and above had significantly higher scale cortisol levels compared to the control.

Table 9: The mean scale cortisol concentrations after subjecting Nile tilapia (*O. niloticus*) to stocking density treatment.

Treatment (Fish / tank**)**	**Mean scale cortisol concentrations (mg/g)**			**Std. Deviation**
CTR 20	2.47^{a}			0.04
25	2.52^{ab}	2.52^{ab}		0.16
40		2.69^{bc}	2.69^{bc}	0.062
55		2.72^{bc}	2.72^{bc}	0.04
70		2.73^{bc}	2.73^{bc}	0.20
85			2.75^{c}	0.14
100			2.76^{c}	0.10

*The superscripts indicates significant difference at $p<0.05$ of the mean scale cortisol concentration following stocking density treatment. Treatment with 40 fish / tank and above had significantly higher scale cortisol levels compared to the control.

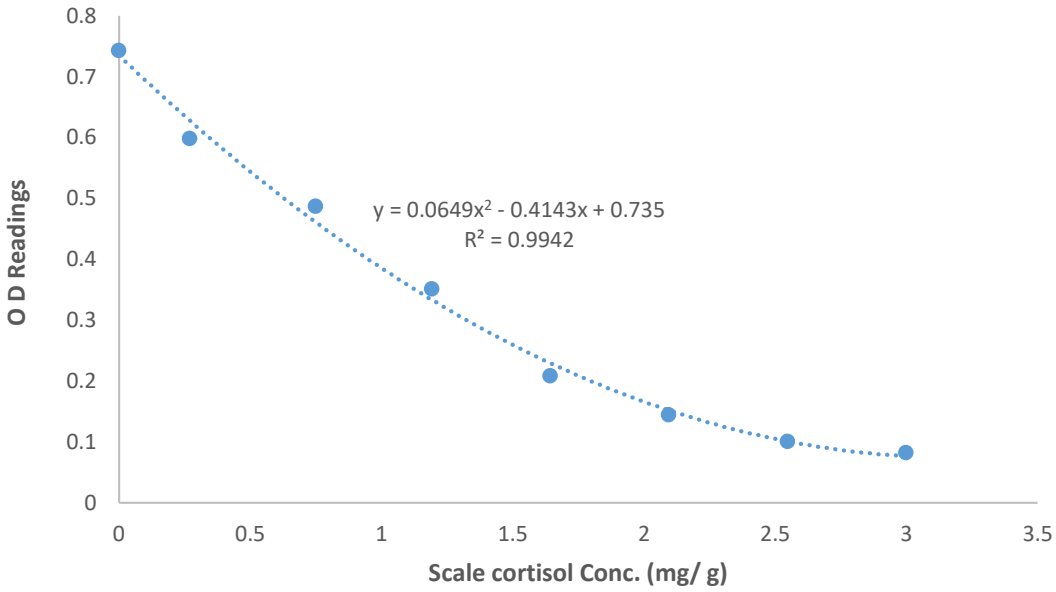

Figure 6: Scale cortisol standard curve used to determine the scale cortisol concentrations in the plasma obtained from Nile tilapia in ammonia and stocking density treatments.

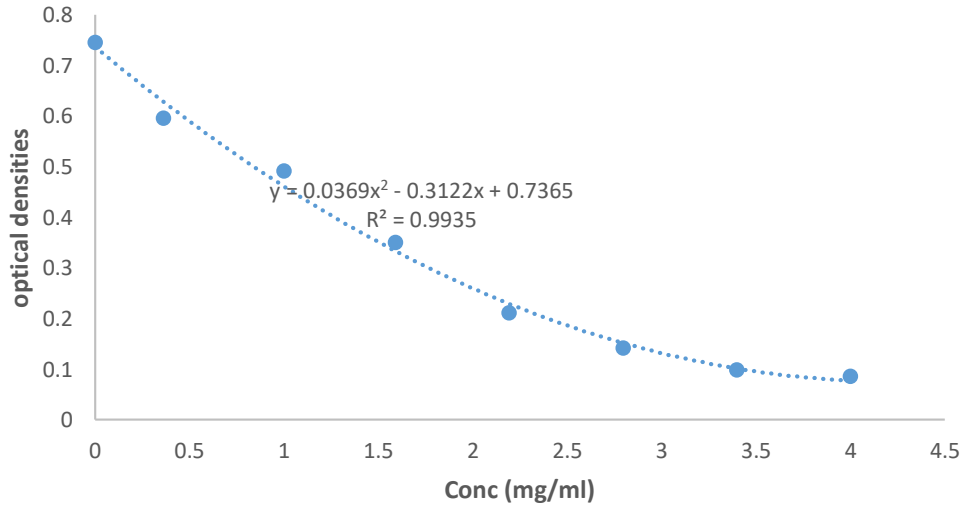

Figure 7: Plasma cortisol standard curve used to determine the plasma cortisol concentrations in blood samples from Nile tilapia in ammonia and stocking density treatments.

4.4 Principal Component Analysis (PCA) of the results from the stocking density and ammonia treatments

Raw data was filtered generating 47,203,186 to 53,152,344 clean reads in the ammonia treatment and 45,860,616 to 54,721,982 in the stocking density treatment. All the samples had Q20 and Q30 value greater than 97.01 and 92.01respectively and a GC content between of 50.01 and 50.95. When compared to the reference genome i.e., from *O. niloticus*, the ratio of the mapped reads was approximately 92%. From the results of the Principal Component analysis (PCA) it is evident that the samples clustered into the controls consistent with the sample grouping (Figure 8 and 9). These results are an indication that the data from this analysis were of high quality and suitable for DEGs and other downstream analysis.

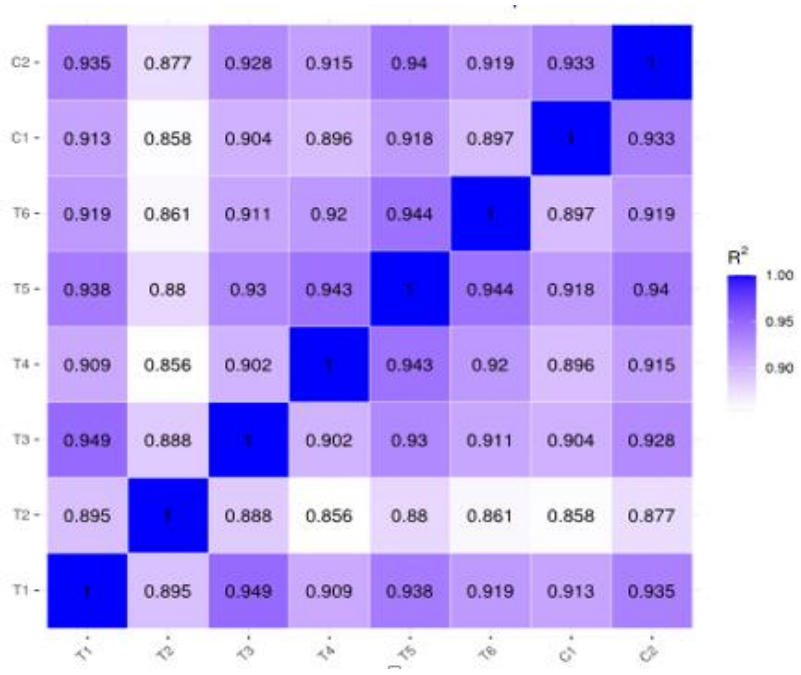

Figure 8: Inter-sample correlation heat map showing the correlation between the *O.niloticus* samples from the ammonia treatment (T1, T2.and T3), stocking density treatment (T4,T5 and T6) and the controls (C1 and C2

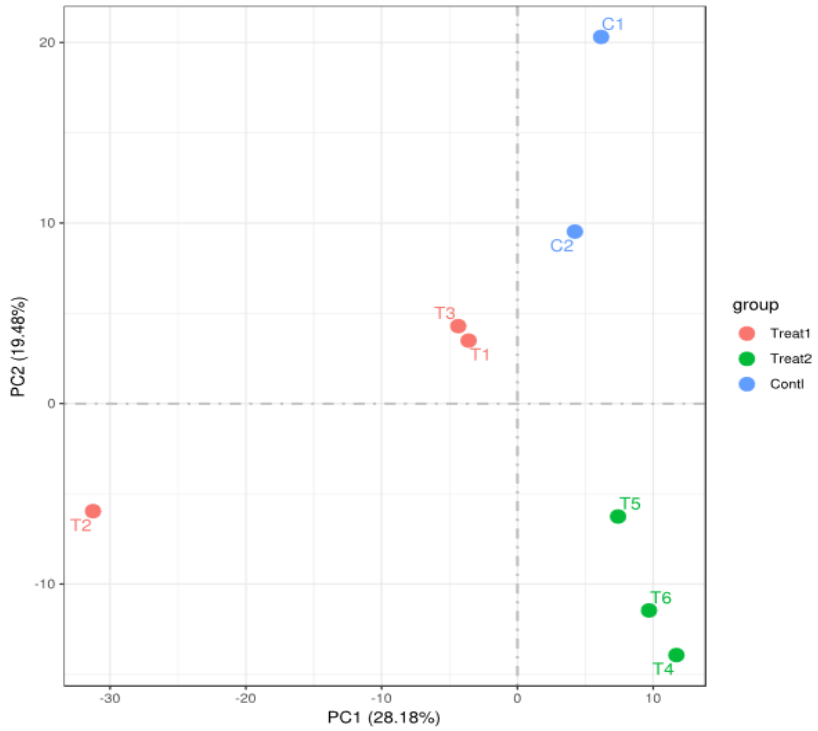

Figure 9: Principal Component Analysis showing the clustering of the *O.niloticus* samples from the ammonia treatment and the stocking density treatment.

4.5 Differential Gene Expression

To identify the regulated genes, volcano plots were used. A combined volcano plot for the ammonia and stocking density treatments revealed myosin light chain 13, myosin 7 and acyl-coenzyme thioesterase 4 as the most significantly up-regulated genes while potassium voltage-gated channel subfamily a regulatory beta subunit 1b was the most enriched gene (Figure 10).

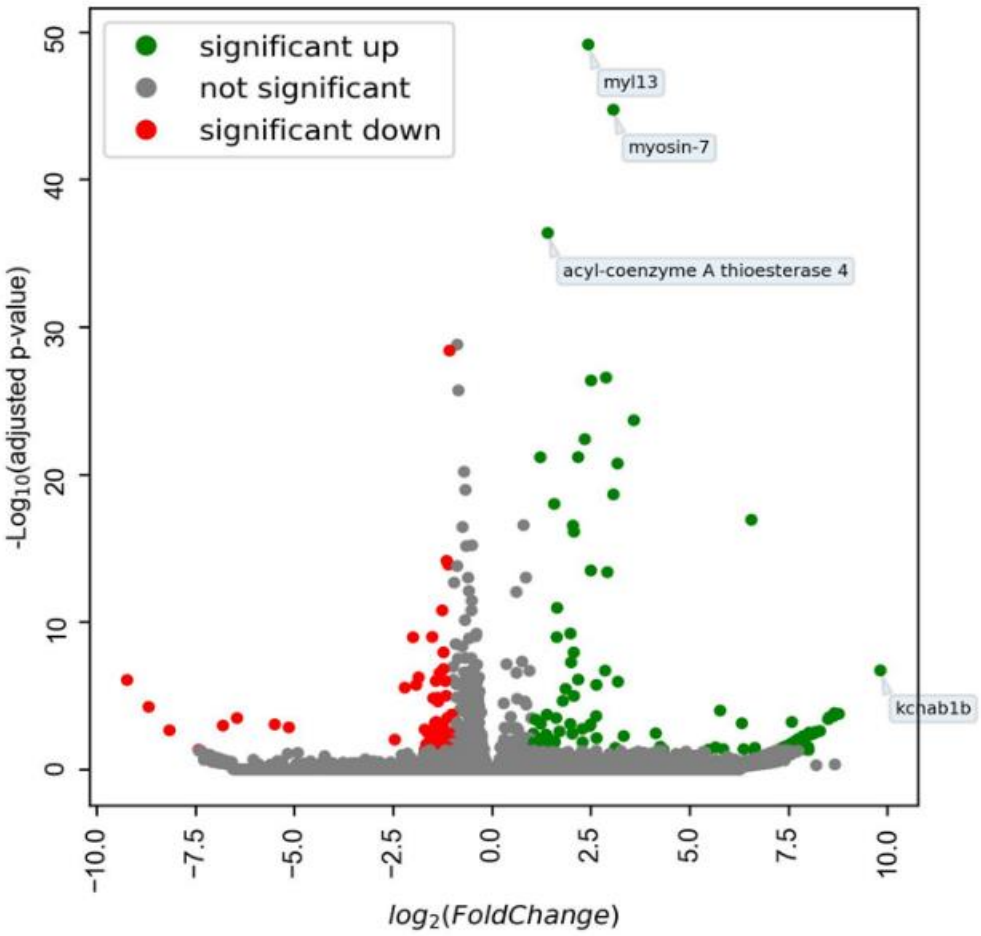

Figure 10: combined volcano plot showing the up and down regulated genes in both ammonia and stocking density treatments. The red dots represent the down regulated, the green dots represent the up regulated while the grey dots represent those that are not significantly regulated in the two.

To identify the up and down regulated genes in the ammonia treatment, a volcano plot was drawn. A total of 20,483 genes were identified from the ammonia treatment samples out of which 156 genes were up regulated and 53 were down regulated (Figure 11).

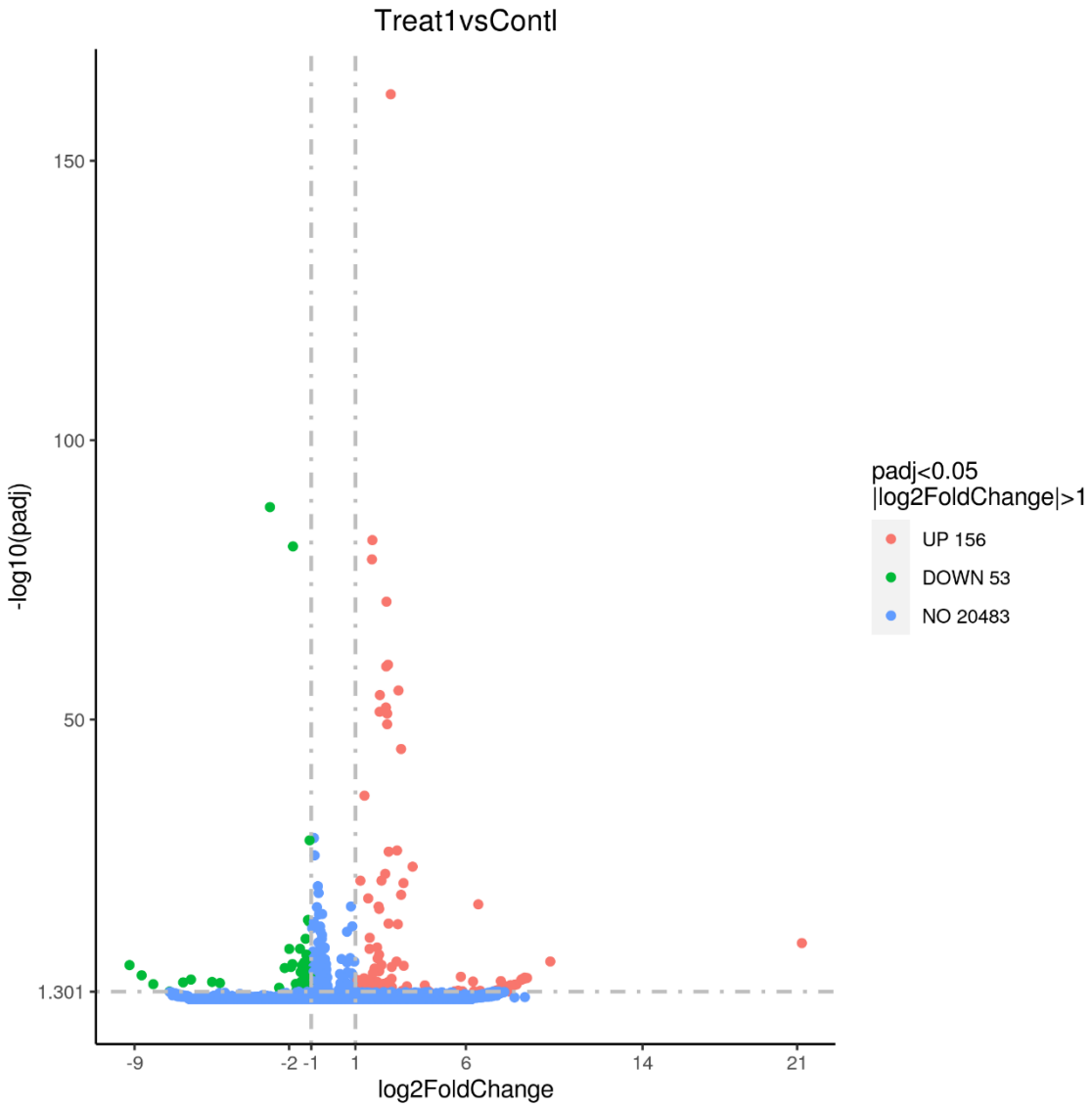

Figure 11: Volcano plot representing the up and down-regulated DEGS in ammonia treatment. The green dots represent the down regulated genes, the red dots depict the up-regulated genes and the blue ones represent the genes that are not significantly up or down regulated under high ammonia concentrations

On the other hand, a volcano plot for the stocking density treatment revealed 19,781 genes with 175 genes being up regulated and 77 genes being down regulated (Figure12).

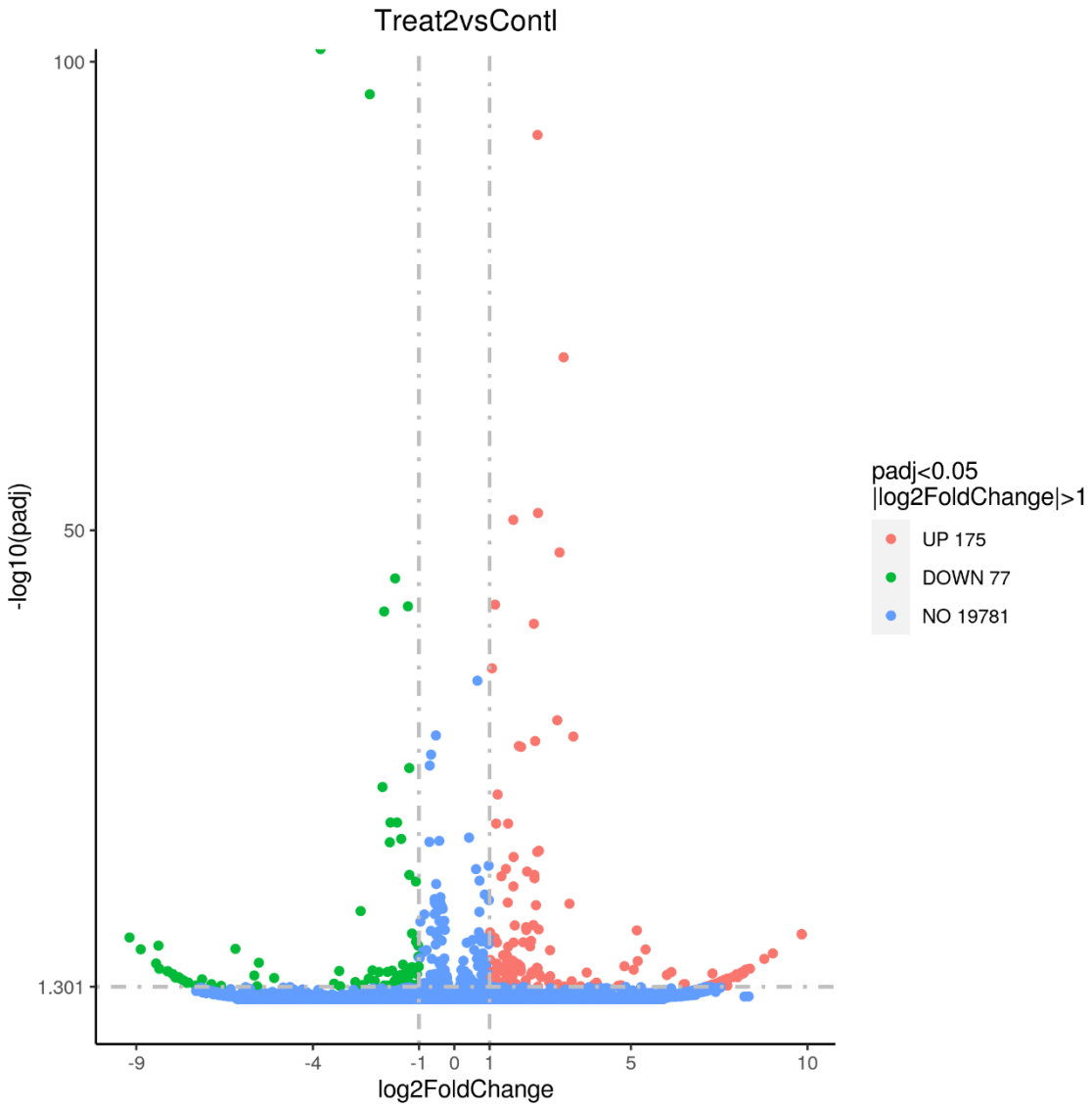

Figure 12: Volcano plot representing the up and down-regulated DEGS in stocking density treatment. The green dots represent the down regulated genes, the red dots depict the up-regulated genes and the blue ones represent the genes that are not significantly up or down regulated under high ammonia concentrations.

Comparative transcriptome analysis revealed the proportion of regulated genes. Ammonia treatment had a total of 209 Differentially Expressed Genes (DEGs) with 156 being up-regulated and 53 down-regulated. On the other hand, stocking density treatment had 252 DEGs of which 175 were up-regulated and 77 down-regulated (Figure13).

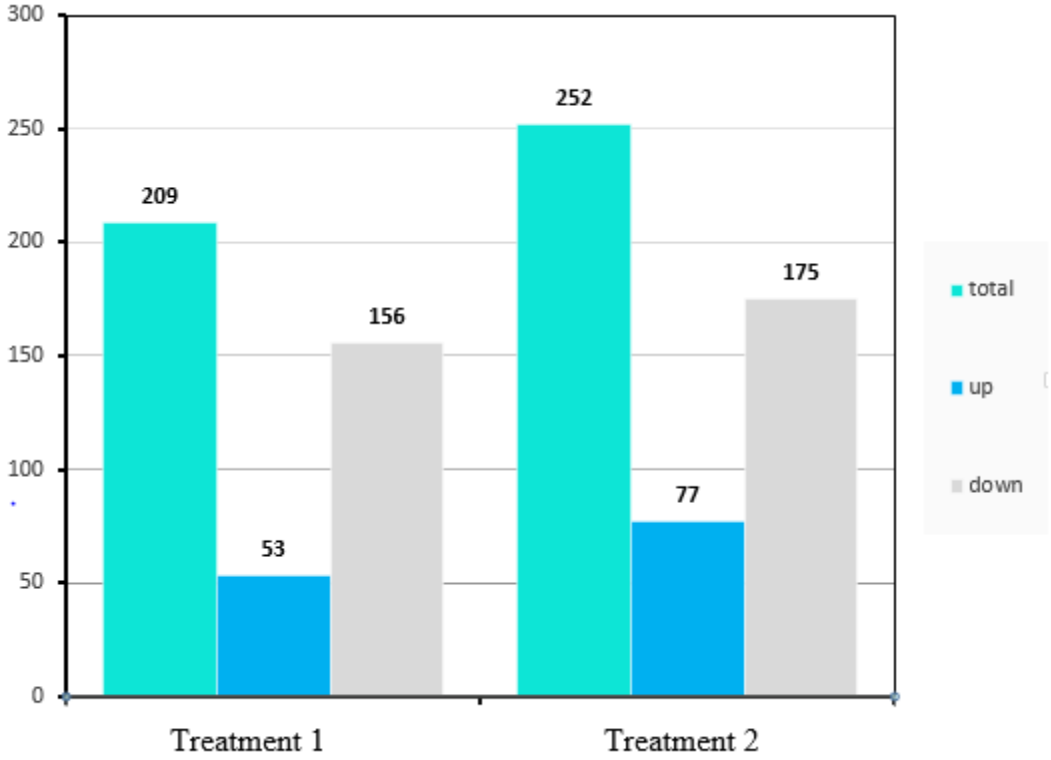

Figure 13: Differentially expressed genes in ammonia (treatment 1) and stocking density (treatment 2) compared to the controls. The threshold for a gene to be considered differentially expressed was DESeq2 padj<=0.05 and |log2FoldChange|>=1.0.

Among the up-regulated genes, 132 genes were unique to ammonia treatment, 151genes were unique to stocking density while 24 of them were common to both treatments (Figure14 a). On the other hand, considering the down-regulated genes the ammonia treatment had 36 unique genes, stocking density treatment had 60 while 17 down-regulated genes were common to both treatments (Figure14 b).

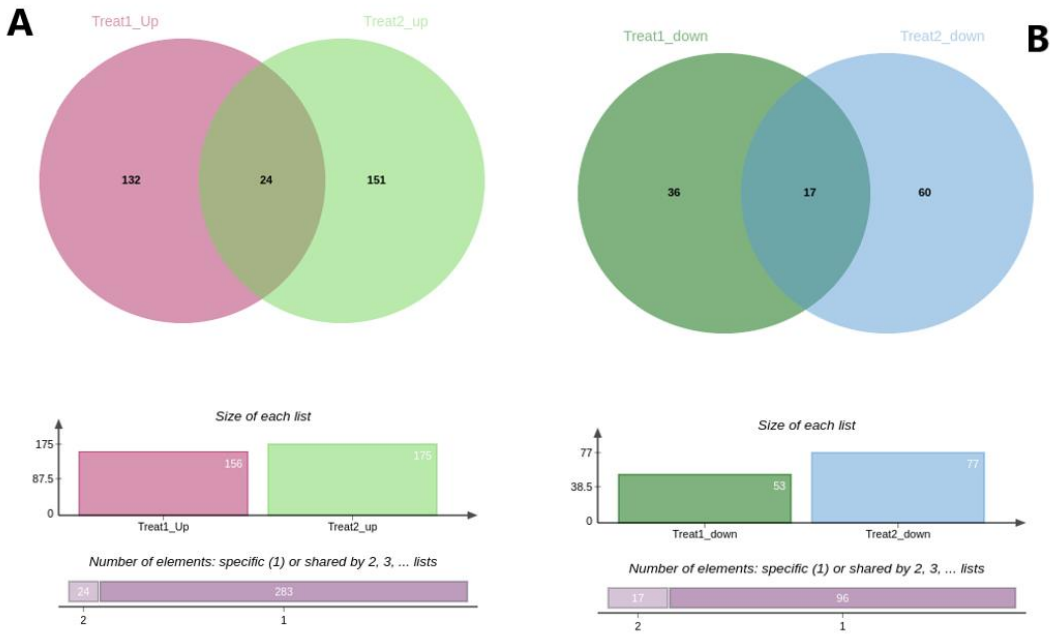

Figure 14: Venn diagram displaying the number of genes that were up- and down-regulated in the ammonia and stocking density treatments. (A) Up-regulated genes in ammonia and stocking density treatment. Pink color represents ammonia treatment, Green represents stocking density while the intersection represents the common elements. (B) Down regulated genes in ammonia and stocking density treatment. Green colour represent ammonia treatment, blue colour represents stocking density treatment while the intersection represents the common elements.

4.6 Gene Ontology analysis of the differentially expressed genes

Gene ontology was performed to classify the DEGs according to their functions. The findings of the GO enrichment study of the DEGs, were divided into 3 GO categories based on Biological processes (BP), Cellular components (CC) and Molecular function (MF). The significantly up-regulated DEGs were mainly enriched in “Oxidation reduction processes” of BP, “cytoskeletal part” and “cytoskeleton” of CC and “motor activity” of MF (Figure15).

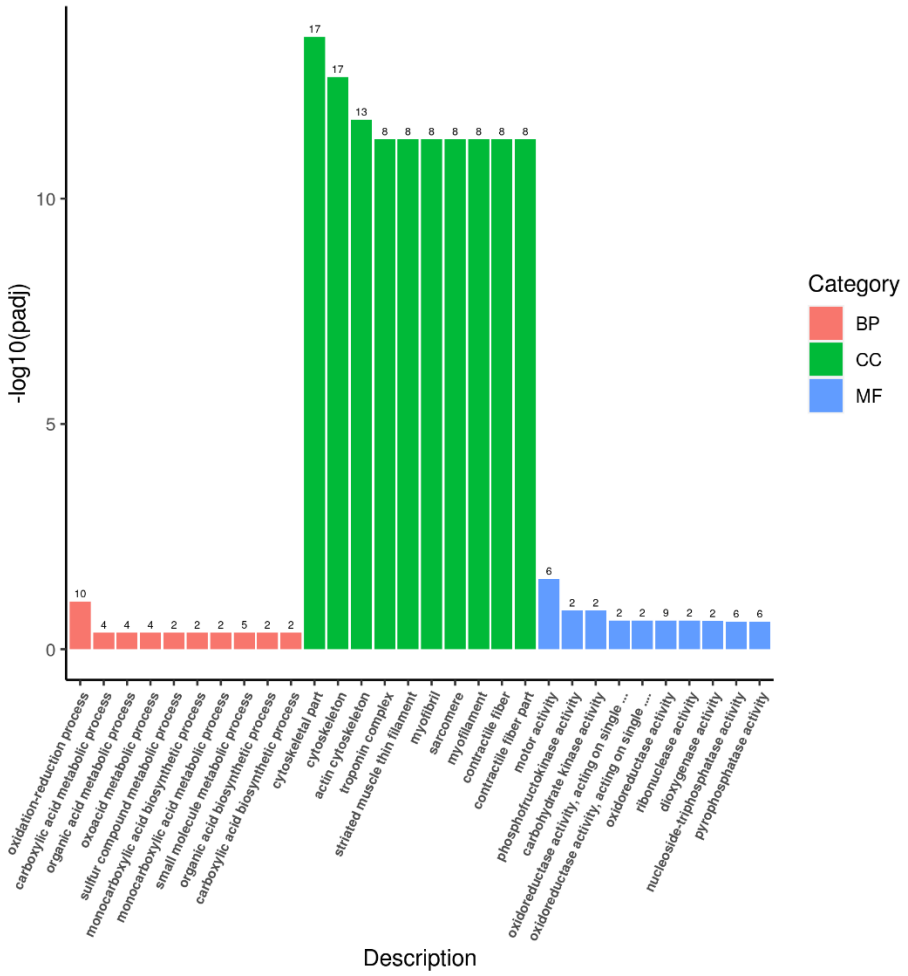

Figure 15: Bar graph representing Gene ontology functional enrichment analysis for the differentially expressed genes under chronic stress. The 10 most up-regulated DEGs in each of the Biological Processes, Cellular Components and Molecular Function categories in ammonia treatment.

On the other hand the significantly down regulated DEGs were mainly enriched in "Nucleosome assembly" of BP, "Nucleosome" of CC and "protein kinase activity" of MF in ammonia treatment (Figure16).

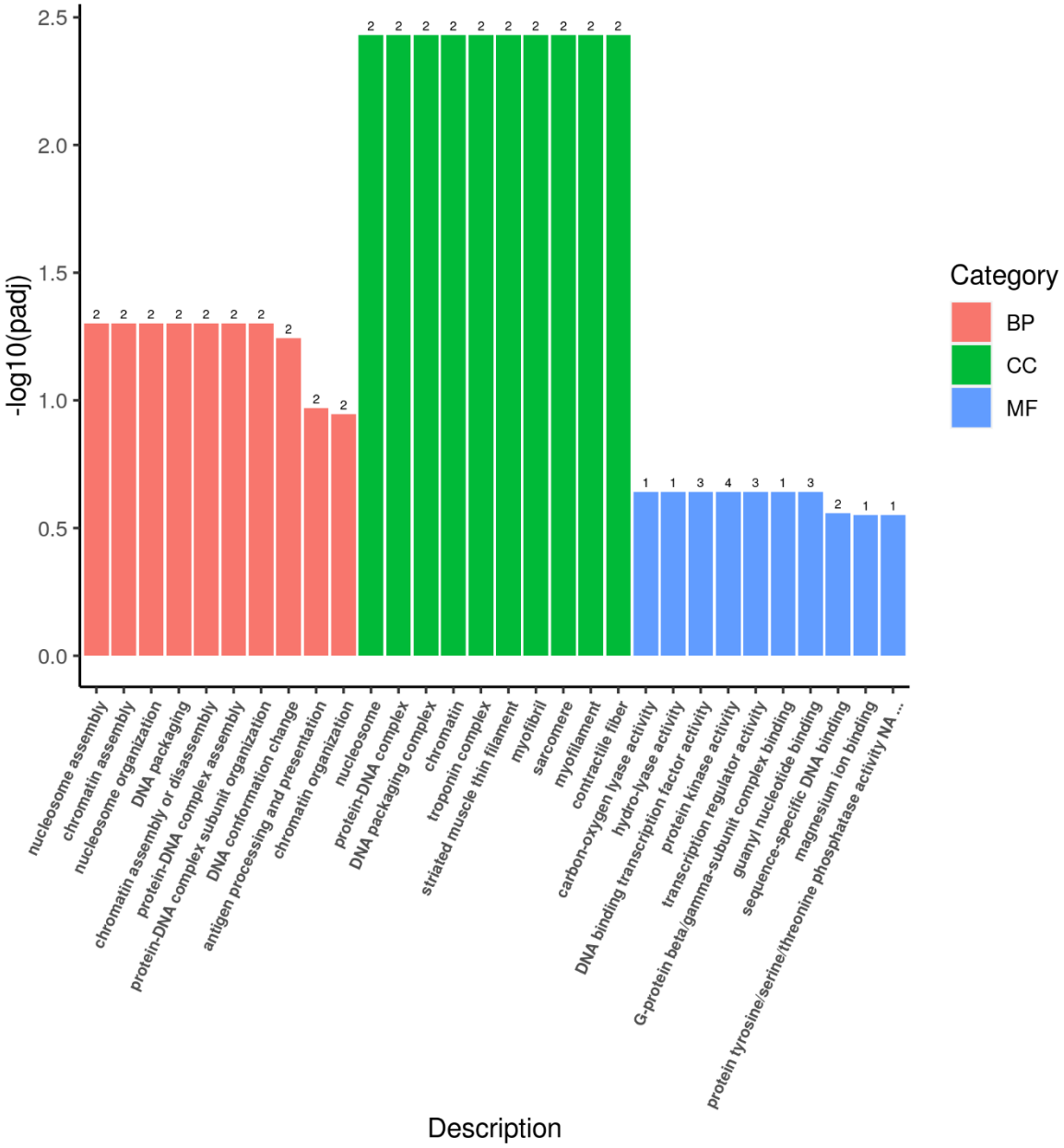

Figure 16: Bar graph representing Gene ontology functional enrichment analysis for the differentially expressed genes under chronic stress. The 10 most down-regulated DEGs in each of the Biological Processes, Cellular Components and Molecular Function categories in ammonia treatment.

The up-regulated DEGs were mainly enriched in “regulation of metabolic process” of BP, “MHC protein complex” of CC and “Sequence specific DNA binding" of MF in the stocking density treatment (Figure17).

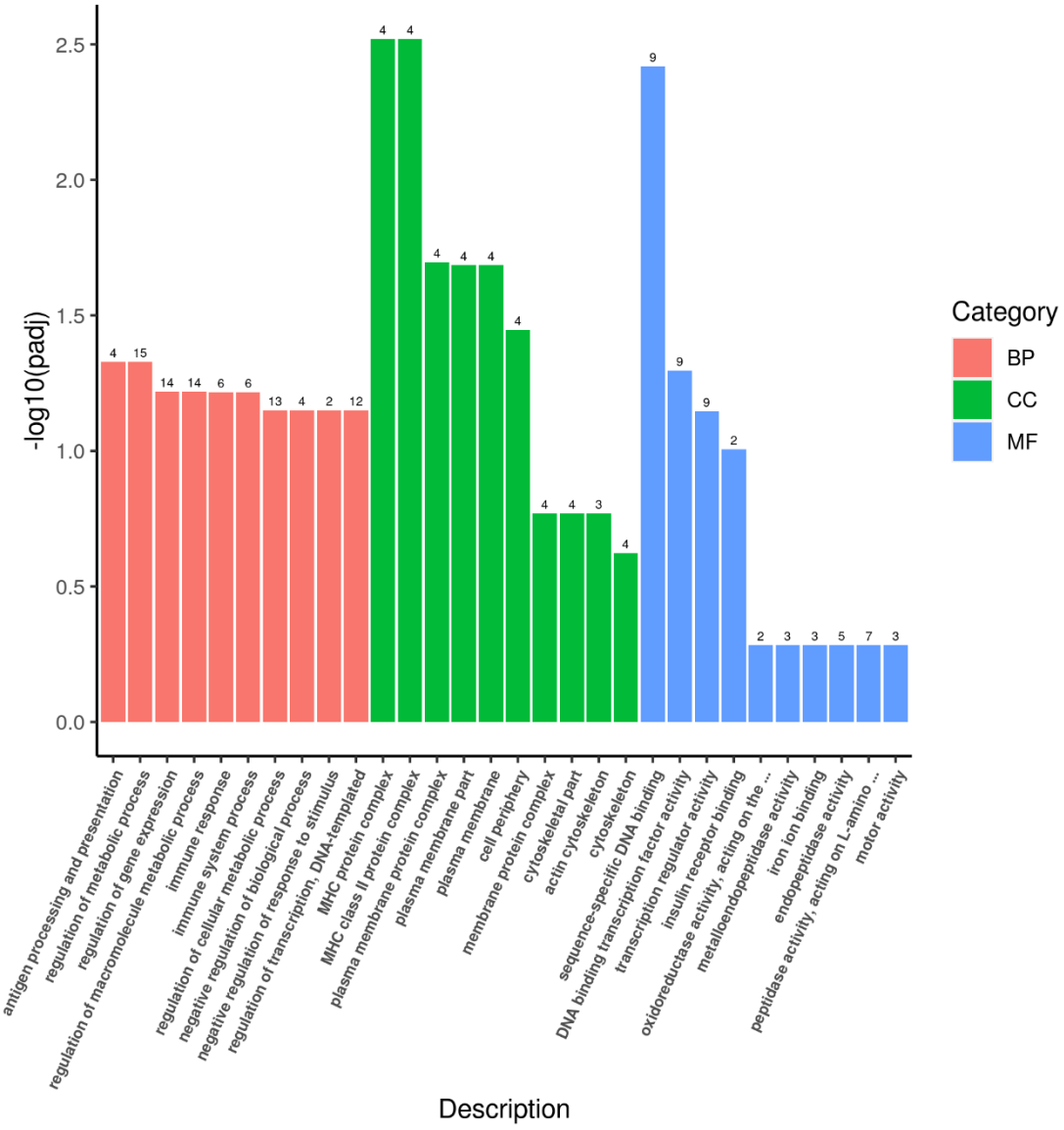

Figure 17: Bar graph representing Gene ontology functional enrichment analysis for the differentially expressed genes under chronic stress. The 10 most up-regulated DEGs in each of the Biological Processes, Cellular Components and Molecular Function categories in the stocking density treatment.

On the other hand the down regulated DEGS were “nucleosome assembly” of BP, “nucleosome” of CC and “Oxygen binding” of MF in the stocking density treatment (Figure 18).

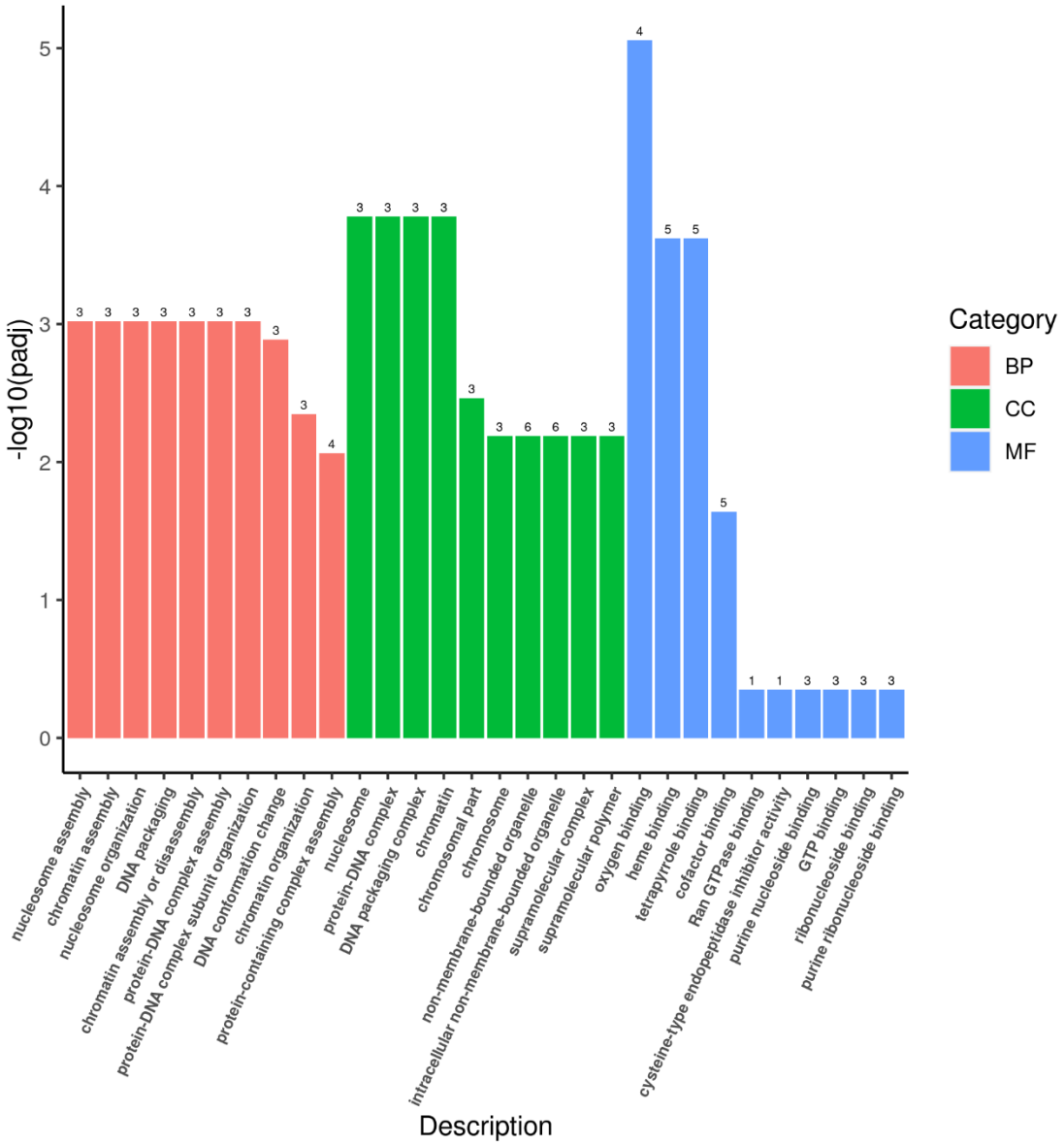

Figure 18: Bar graph representing Gene ontology functional enrichment analysis for the differentially expressed genes under chronic stress. The 10 most down-regulated DEGs in each of the Biological Processes, Cellular Components and Molecular Function categories in the stocking density treatment.

The most enriched pathways were mainly “Oxidation reduction processes” and “carboxylic acid” of BP, “cytoskeletal part I” and “cytoskeleton” of CC and “motor activity” and “pyrophosphate activity” of MF in ammonia treatment (Figure19).

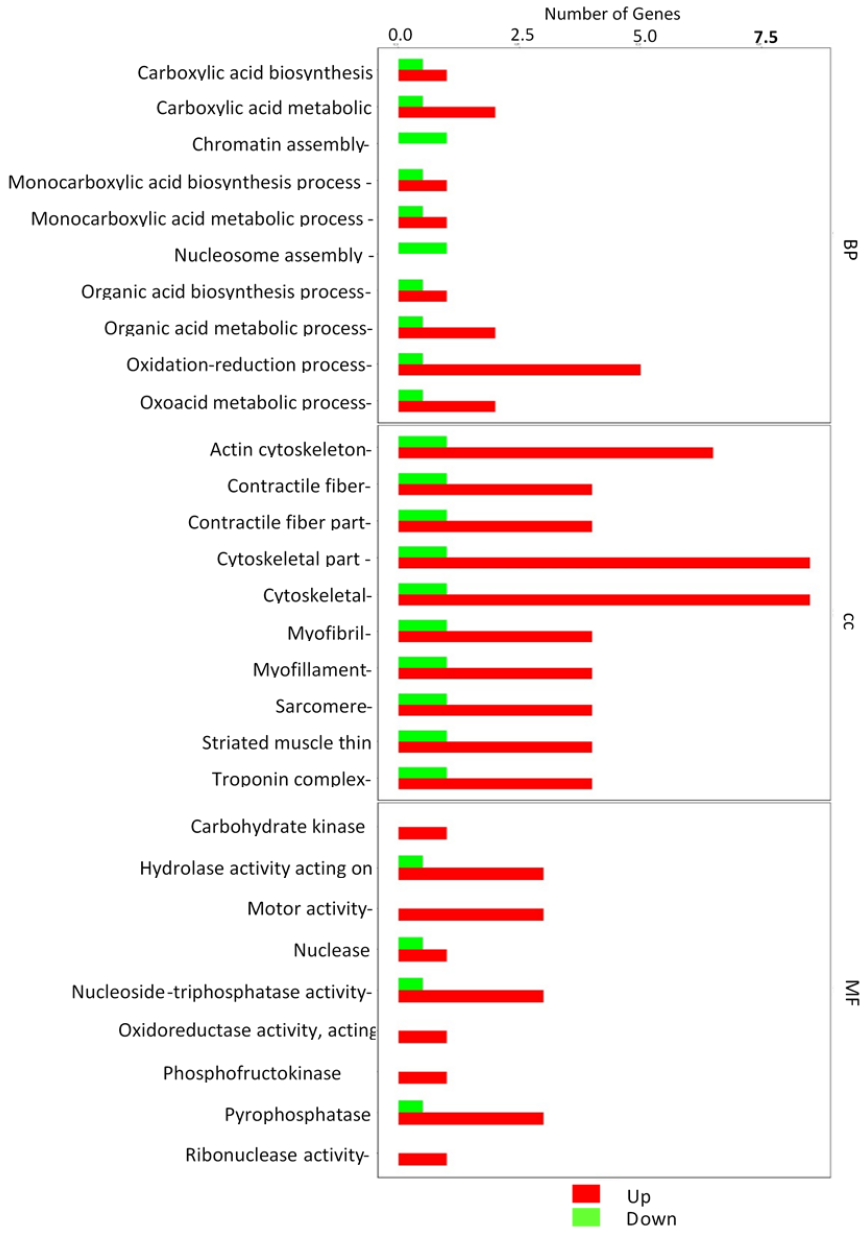

Figure 19: Bar graph representing the most significantly enriched pathways in the ammonia treatment.

On the other hand “immune response” and “immune system process” of BP, “MHC class II” and “MHC protein complex” of CC and “Sequence specific DNA binding” and “Transcriptome regulation activity” of the MF were the most enriched pathways in the stocking density treatment (Figure 20).

Figure 20: Bar graph representing the most significantly enriched pathways in the stocking density treatment.

To understand the function of the significant DEGs in the signaling pathways, the significantly up and down regulated DEGs were annotated the in the ammonia and stocking density treatments in the KEGG database.

To better understand the DEGs in the individuals treated to the two treatments, the ratio between the level of enrichment between the up regulated and the down regulated genes in the three categories was compared. In ammonia treatment immune response of MF, MHC protein complex of CC and sequence specific DNA binding of BP showed the highest difference (Figure18). In the ammonia treatment, oxidation reduction process of MF, cytoskeleton of CC and motor activity of BP showed the highest difference (Figure 19)**.**

4.7 KEGG Analysis

To identify the significantly enriched pathways, the identified DEGs were then subjected to KEGG analysis. The most significantly enriched and up regulated pathways in ammonia treatment were calcium signaling pathway, adrenergic signaling in cardiomyocytes and cardiac muscle contraction (Figure 21). On the other hand the significantly enriched and down regulated pathways in the ammonia treatment was the MAPK signaling pathway (Figure 22).

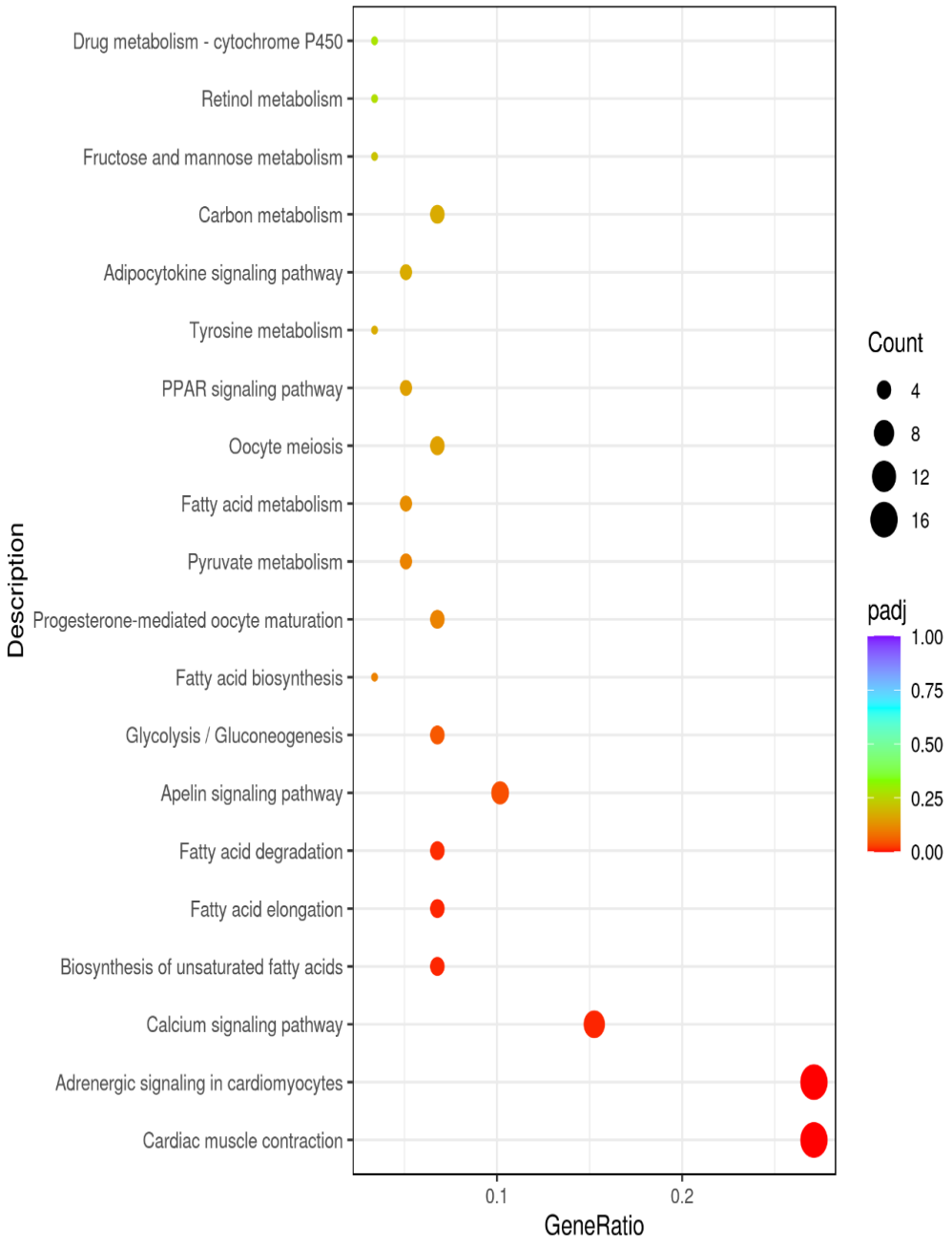

Figure 21: Dot plots representing the KEGG analysis of the most significantly enriched up regulated pathways in stocking density treatment.

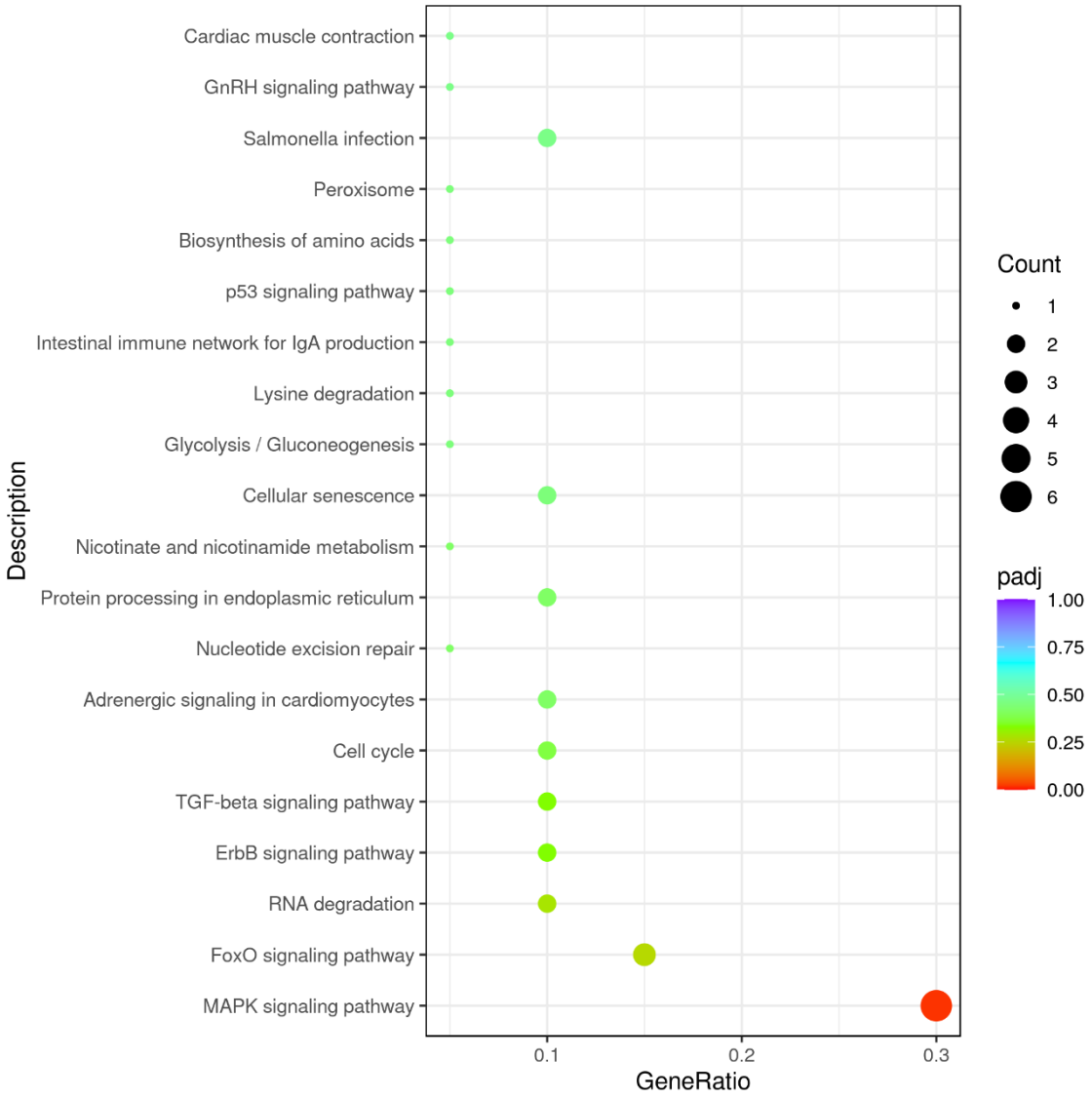

Figure 22: Dot plots representing the KEGG analysis of the most significantly enriched down regulated pathways in ammonia treatment.

Stocking density treatment showed the intestinal immune network for IGA production and phagosome as the most significantly enriched and up regulated pathways (Figure 23) while the fatty acid elongation, biosynthesis of unsaturated fatty acids and the cardiac muscle contraction pathways were the most significantly enriched and down regulated pathways (Figure 24).

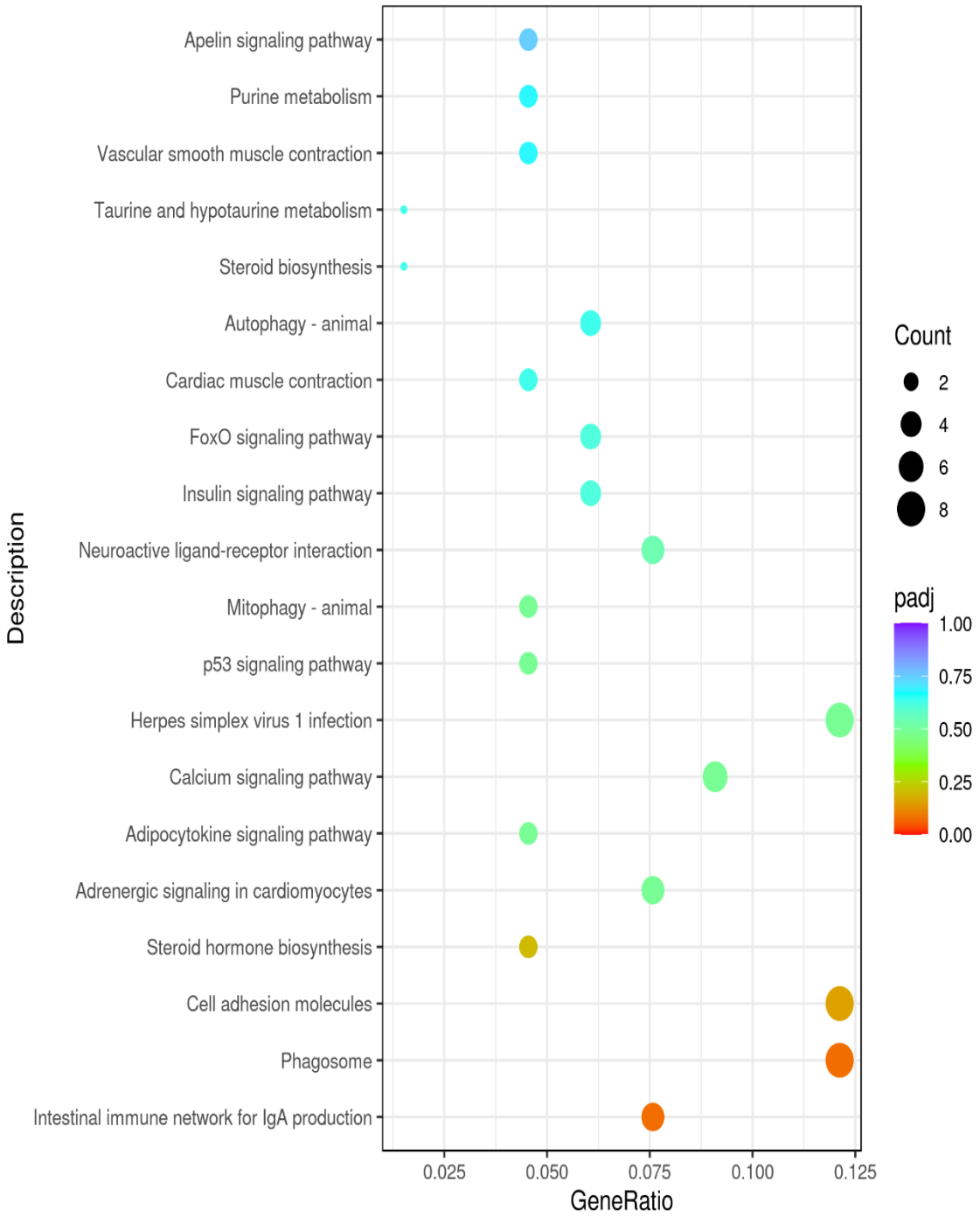

Figure 23: Dot plots representing the KEGG analysis of the most significantly enriched up regulated pathways in the stocking density treatment.

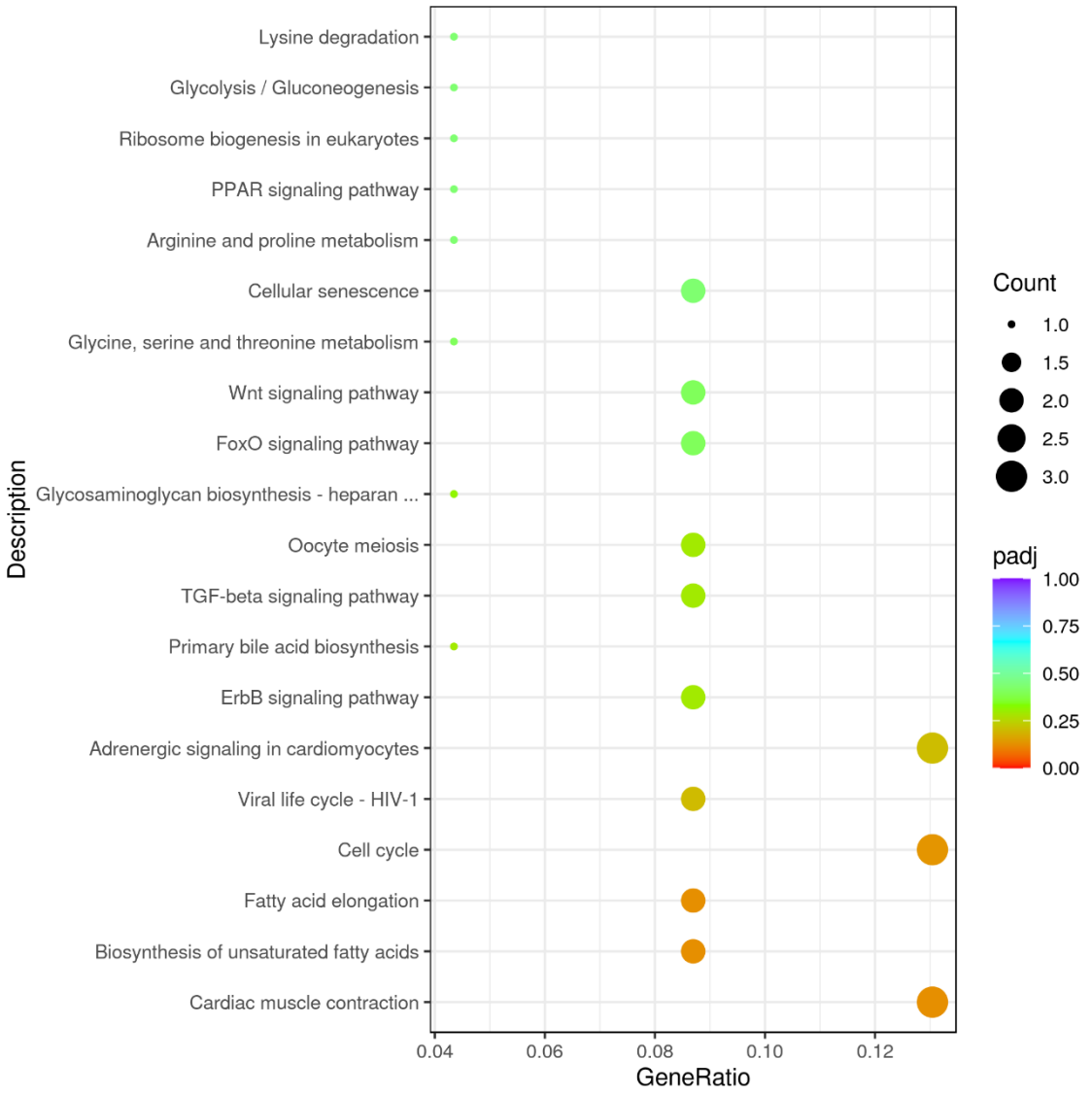

Figure 24: Dot plots representing the KEGG analysis of the most significantly enriched down regulated pathways in the stocking density treatment.

4.7.1 MAPK Signaling Pathway

MAPK signaling pathway was significantly down regulated in ammonia treatment (Figure 25). Six genes in this pathway were significantly enriched following ammonia treatment (Padj ≤0.05) i.e. DUSP1 (dual specific protein phosphatase 1), NHR38 (Nuclear Hormone

Receptor), HSP 72 KDa Protein 1(heat shock protein), myelocytomatosis oncogene homologue (Mych), Growth arrest and DNA damage inducible alpha a (GADD 45aa) and mytogenic activated protein kinase kinase 4 (MAP2K4). Twenty-nine other genes in this pathway on the other hand down regulated but not significantly so (Padj. ≥0.05).

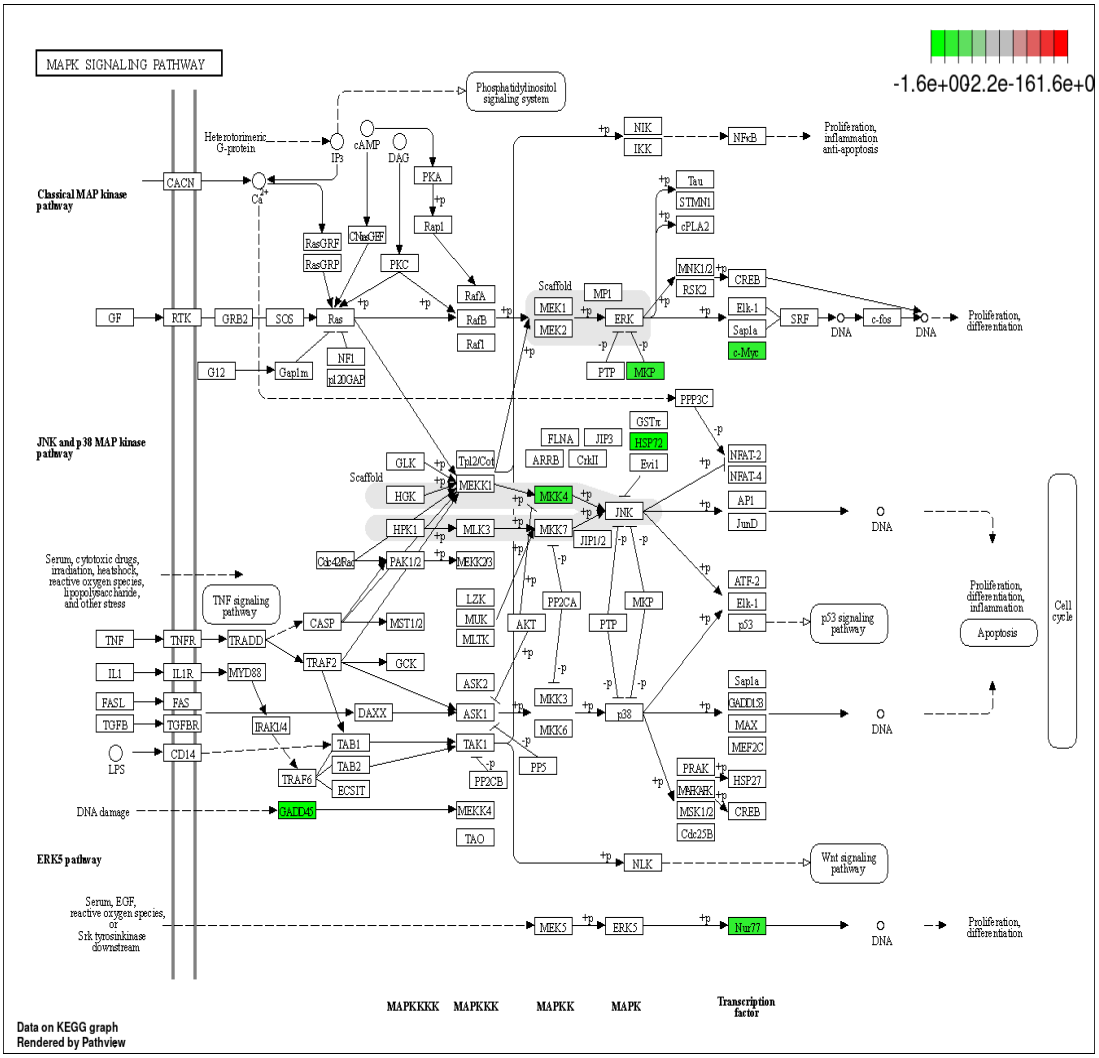

Figure 25: MAPK signalling pathway showing the sequence of phosphorylation leading to the activation of the respective MAPKs. The green colour indicates the significantly enriched genes under chronic stress in *O. niloticus*. The grey colour indicates the protein scaffolds that controls the phosphorylation of the MAPKs.

4.7.2 FOXO Signaling Pathway

The FOXO signaling pathway was significantly down regulated in ammonia treatment (Figure 26). Three genes in this pathway were significantly enriched following ammonia treatment (Padj ≤0.05) I.e. BCL6Atranscription repressor a, BCL6Atranscription repressor b and Gadd45aa- growth arrest and DNA – damage – inducible , alpha ,a. (Figure 26).

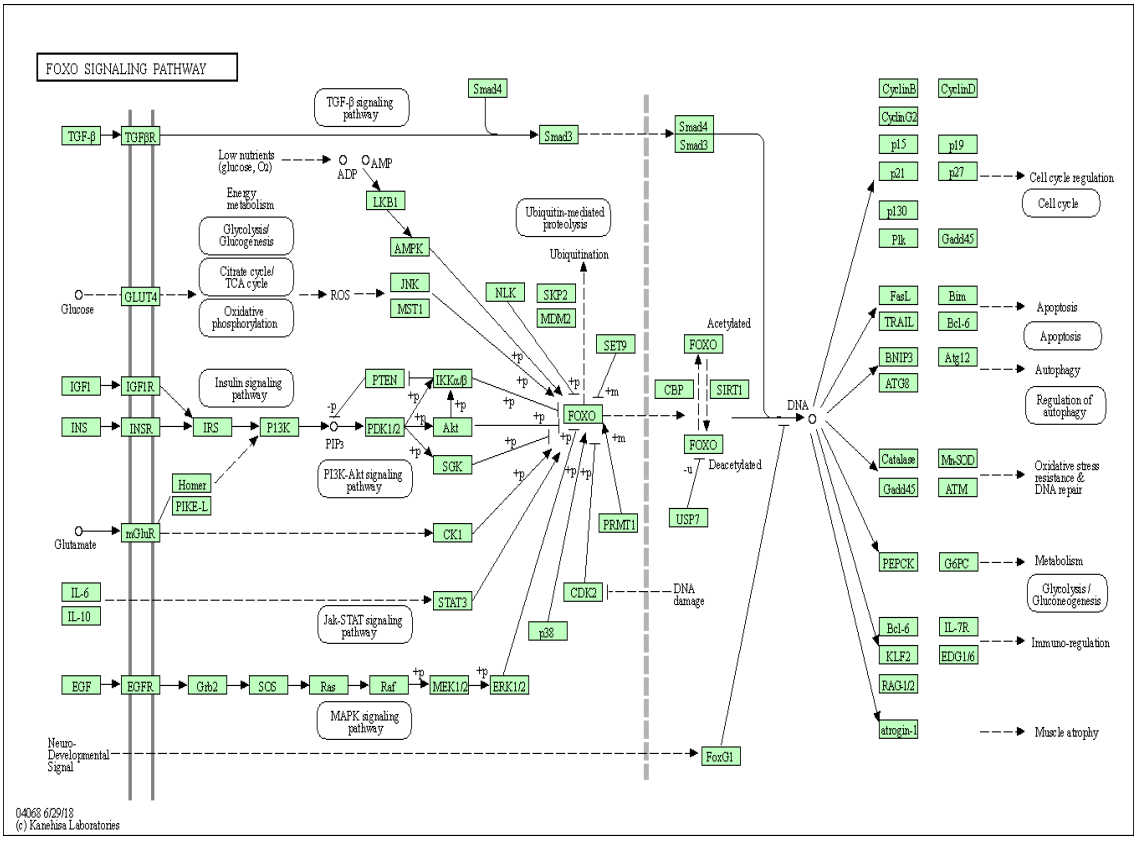

Figure 26: FOXO signaling pathway showing the sequence leading to the acetylation and eventual phosphorylation of FOXO proteins.

KEGG pathways were used to show the position of the DEGs in the metabolic pathways to understand their function in phenotype related to growth performance. The significantly enriched DEGs were mainly involved in muscle activity, energy mobilization and immune related functions (Figure 27, 28 and 29).

4.7.3 Cardiac Muscle Contraction Pathway

The cardiac muscle contraction pathway is responsible for the contractility of the heart and consequent pumping motion. This pathway was significantly up-regulated in Ammonia treatment. Ammonia induced stress up-regulated 16 DEGs in this pathway while down regulating one DEG in the same. In stocking density treatment, the same pathway was up-regulated with 16 DEGs being up and 1 DEG being down regulated (Figure 27).

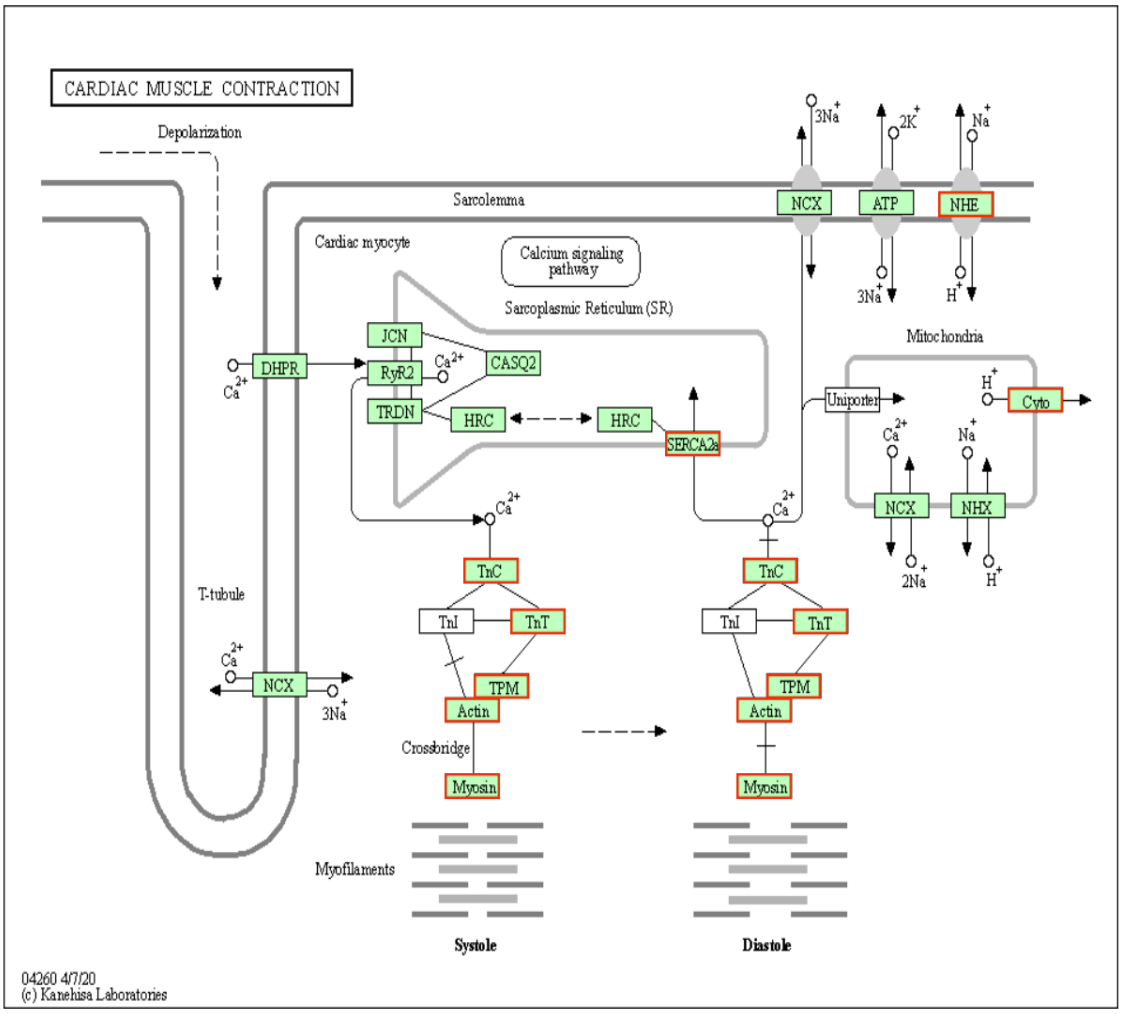

Figure 27: The cardiac muscle contraction pathway showing the pathway terms that are enriched in ammonia treatment. The red border shows the enriched terms.

4.7.4 Adrenergic Signaling in Cardiomyocytes Pathway

Among the downstream effects of the activation of the adrenergic signaling pathway is the stimulation of apoptosis, enhanced contractility of cardiac muscles as well as its speed of contraction. This pathway was significantly up-regulated in Ammonia treatment. Ammonia induced stress up-regulated 16 DEGs and down regulated 2 DEGs in this pathway (Figure 28).

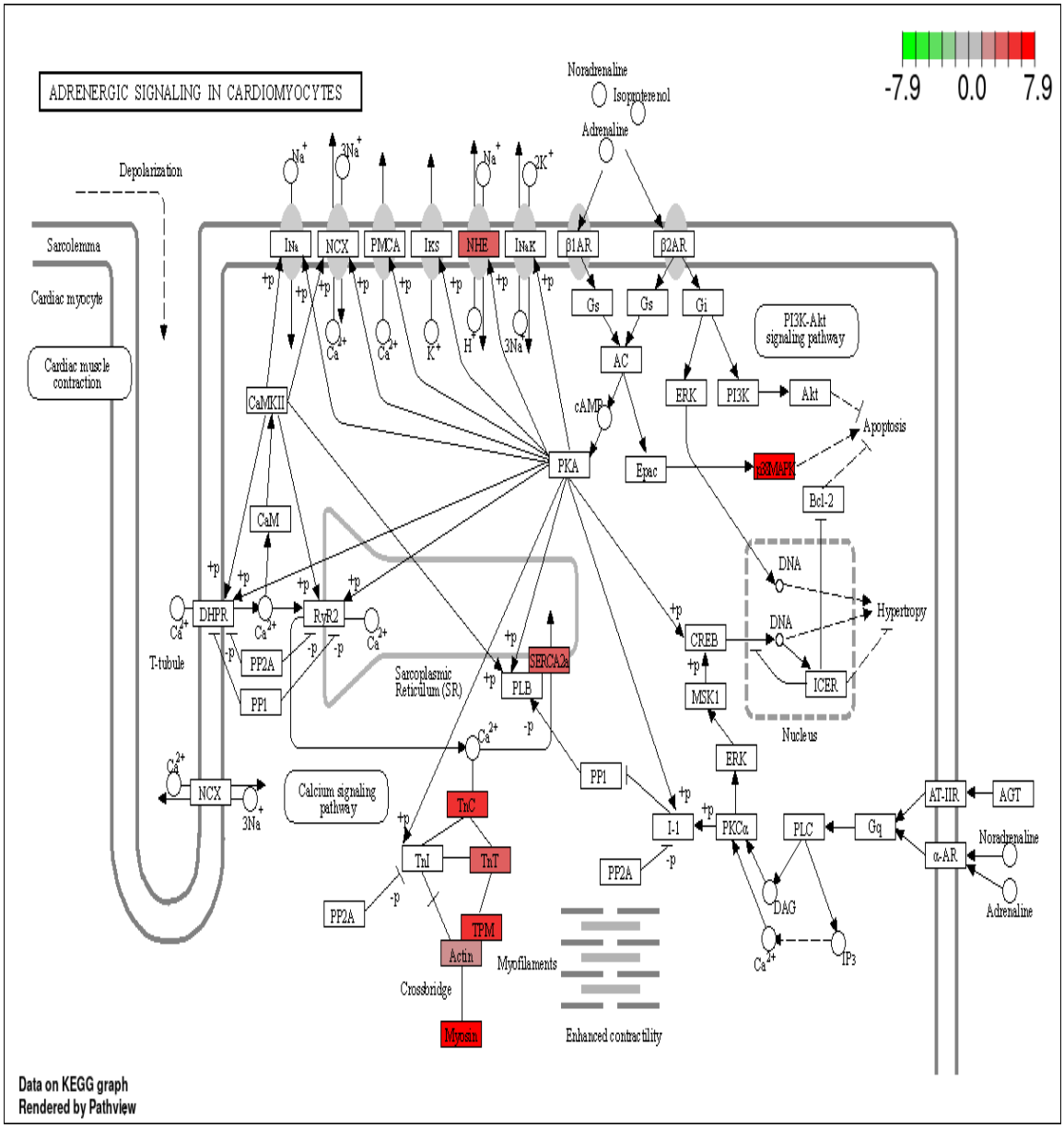

Figure 28: Adrenergic signalling pathway showing the pathway terms that are enriched in ammonia treatment. The colour shades refer to the extent of up regulation

4.7.5 Fatty Acid Degradation

This pathway was significantly up-regulated in Ammonia treatment. Ammonia induced stress up-regulated 4 DEGs in this pathway (Figure 29**)** Namely; acsl1a acyl-CoA synthetase long chain family member 1a, acsl1a acyl-CoA synthetase long chain family

member 1a, cpt1ab carnitine palmitoyltransferase 1Ab (liver) and alcohol dehydrogenase 1

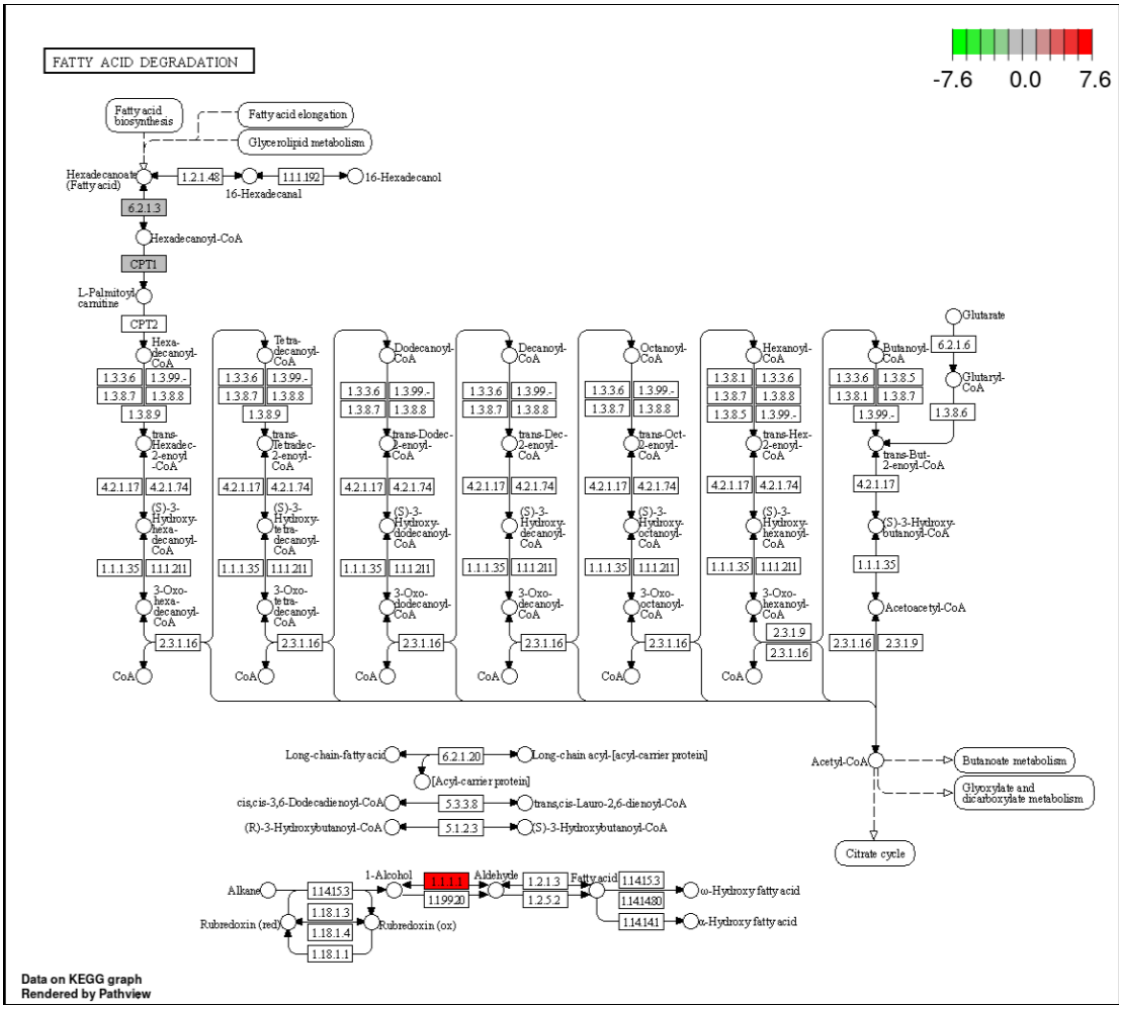

Figure 29: Fatty acid degradation pathway showing the pathway terms that are enriched in ammonia treatment. The color shades refer to the extent of up regulation.

4.8 Validation of RNA-seq Results.

Results from the RT-qPCR showed RT-qPCR and that expression alter in the same way. The RT-qPCR data and the Illumina sequencing findings agreed, demonstrating the validity and accuracy of the Transcriptome analysis (Figure 30). Through this analysis, the results from RNA-seq guarantee that DEGs that were discovered under stress and that

additional research into these or other DEGs from the transcriptome data is viable and sustainable.

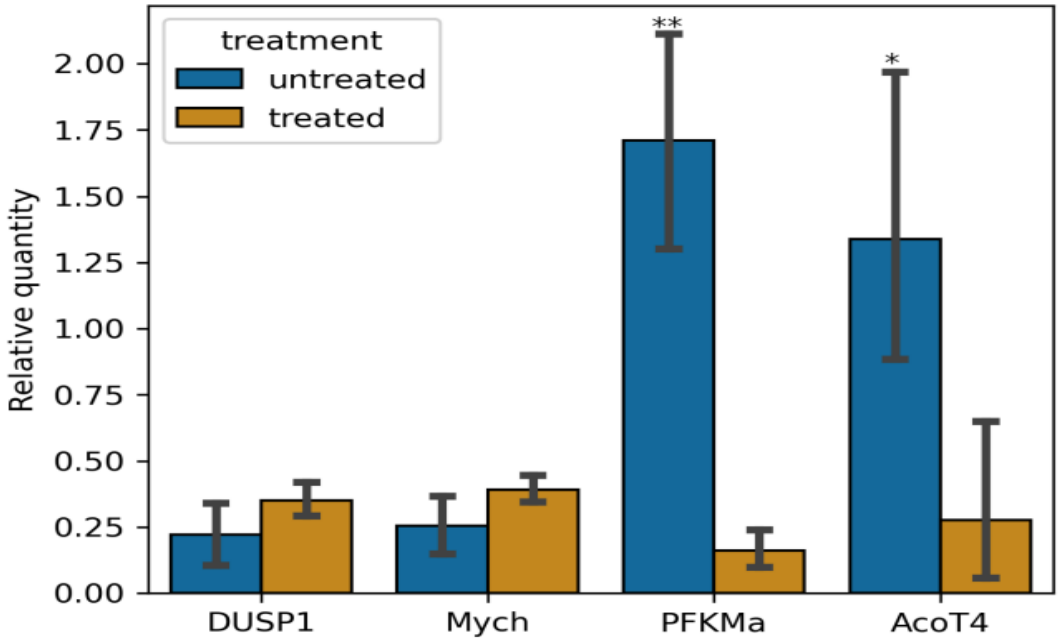

Figure 30: Transcript abundance of DUSP1, Mych, PFKMa, and AcoT4 when Nile tilapia was stressed with a concentration of 0.12 mg/L of ammonia and stocking density of 70 fish per tank measured by RT-qPCR. (one-way ANOVA; * indicates $p \leq 0.05$ while ** indicates $p \leq 0.001$).

4.9 Pan Genomic Analysis of the Kakamega strain

After clustering, 24,159 gene clusters and 465 single-copy orthologs were detected across the 14 teleost species including *B. splendens* (Figure 31)

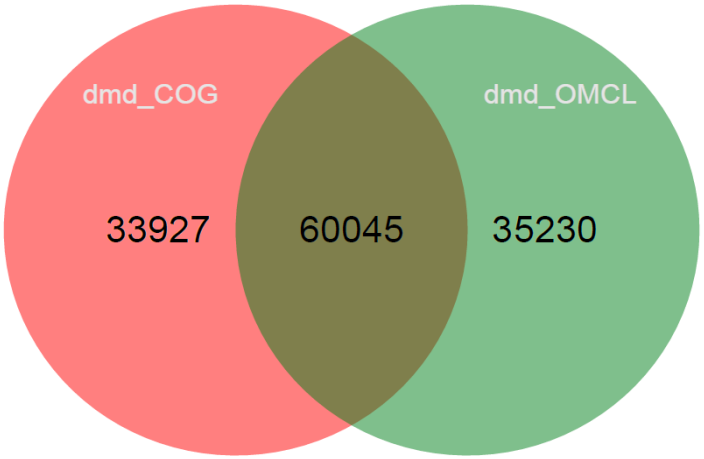

Figure 31: Venn diagram representing the pangenome matrix generated from the intersection between orthologues generated from the Orthologous analysis performed with orthoMCL and COG Triangles programs and a perl script from GET_HOMOLOGUES program. The genomes of the isolates contained 95,275 and 93,972 orthologous groups, respectively, identified by the orthoMCL and COG triangle algorithms, of which 60,045 ortho groups.

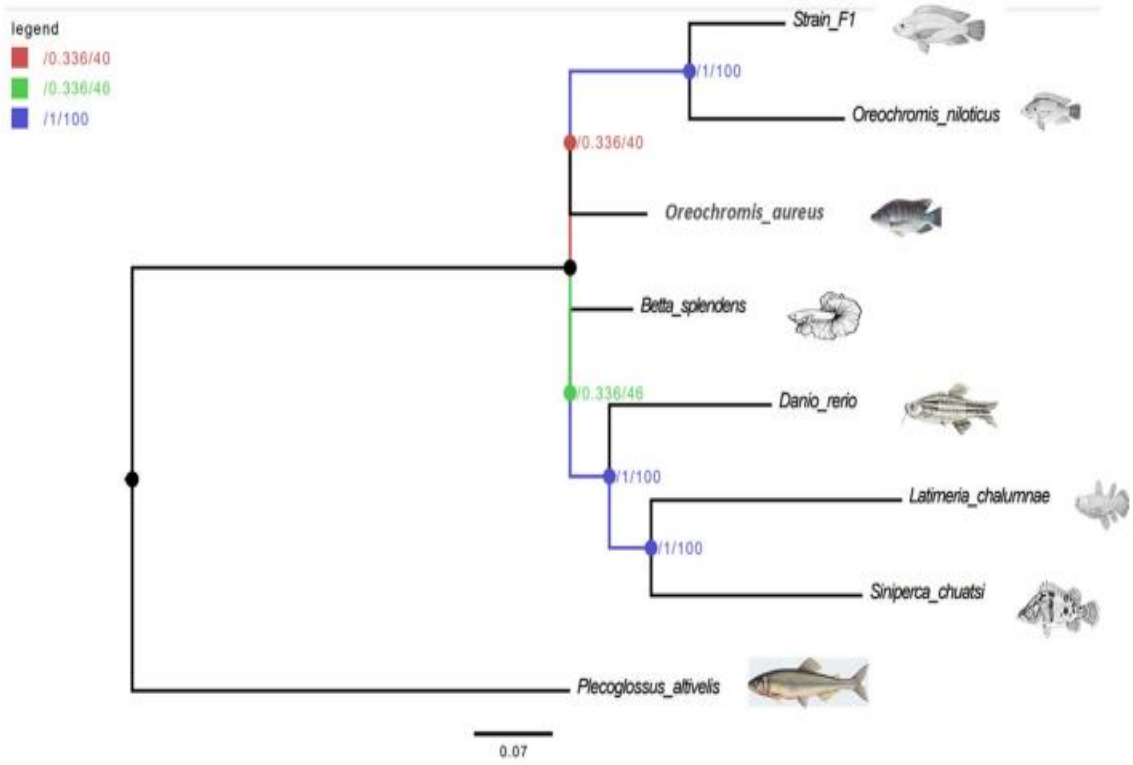

Figure 32: A phylogenomics Fig tree diagram representing the seven species of interest indicating their estimated points of divergence

The divergence of the species was determined and the experimental sample was found to be closely related to *O. aureus* and had diverged together from common ancestor. The phylogenetic analysis revealed a true relative of experimental sample as *O. niloticus (Nile tilapia)* as they clustered into one clade (Figure 32).

A panel of SNPs markers was developed for the identification of six fish species of interest, (*O. niloticus, B. splendens, experimental sample, O. aureus, D. rerio, L. chalumnae*, for comparision purposes. A total of 27,577 expressed sequence tags were collected. From these tags a total of 11,020 genomes had a hit with betta splendens (39.96%), 16,333 with the experimental sample (59.23%), 18,272 with oreochromis niloticus (66.3%) and 11,852

with *O. aureus* (42.98%). The sequence tags expressed were designed from the region having the best homology with the reference genome (Figure 33).

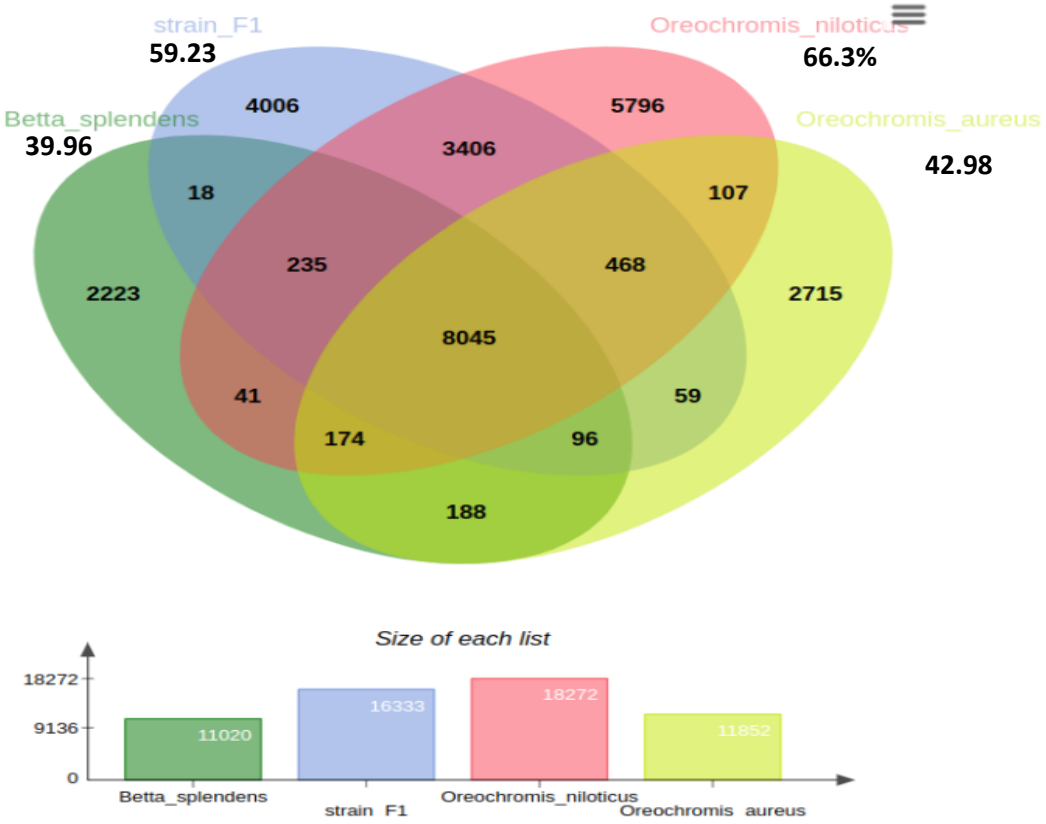

Figure 33: Venn diagram representing the distribution of genes shared by our sample and *O. niloticus*, *B. splendens*, experimental sample and *O. aureus*.

In the Figure 33 above, each ellipse represents a model species and each intersection depicts the number of genes shared by two species or more. For each model species, the number of genes and the percentage of 27,577 genes are indicated. *O. niloticus* had highest number of shared genes with the other species (18,272) amounting to 66.3% while experimental sample followed with 16333 (59.23%) shared genes. *O. aureus* shared 11,852 (42.98%) genes while *B. spendens* shared 11,020 (39.96%) genes with other species. *O. niloticus* had 5,796 unique genes, experimental sample had 4,006 unique genes, *O.aureus* 2,715 and lastly *B. spendens* with 2,223 unique genes.

4.10 SNP Analysis

The number of genotypes and the cumulative number of genotypes (%) called at increasing levels of sequencing depth were identified and plotted on graph as shown in Figure 34.

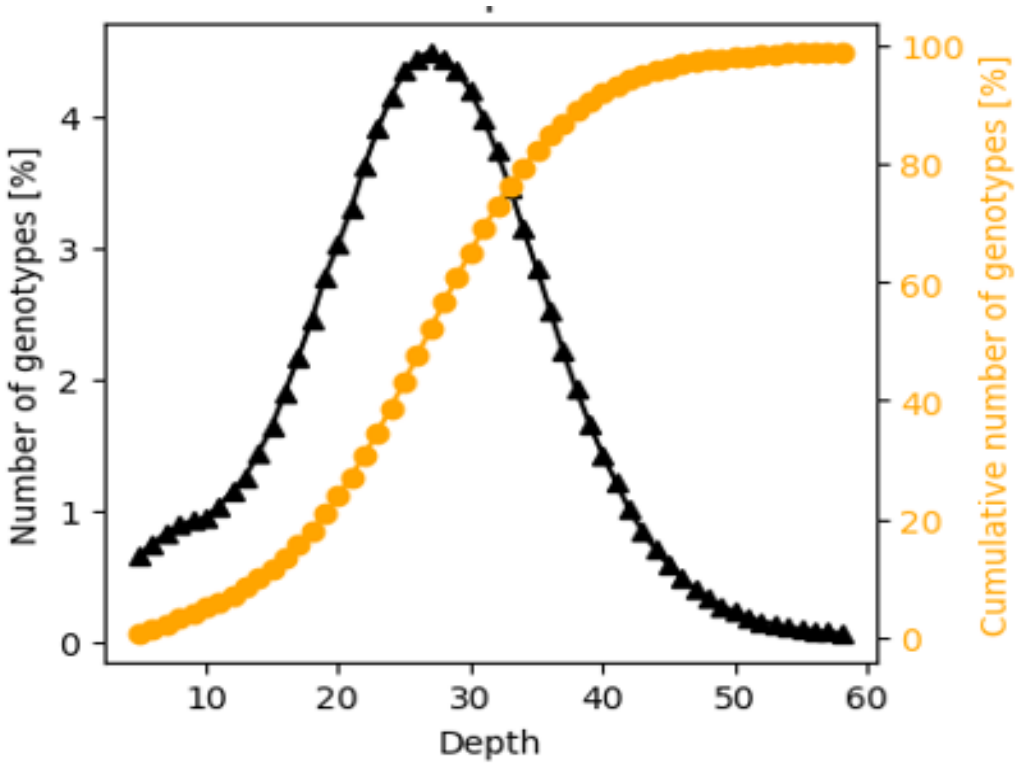

Figure 34: A combined graph representing the SNPs and genotype counts from Genotyping by Sequencing (GBS) data. The black curve displays the Number of genotypes called at increasing levels of SNPs sequencing depths while the orange curve represents the Cumulative number of genotypes called at increasing levels of SNPs sequencing depths.

The total number of transitions (Ts) and transversions (Tv) were investigated. The total number of transversions (Tv) (A/G,C/T,G/A AND T/C) were significantly higher for all the pairs. The frequencies of A/C and T/G, A/T and T/A, C/A and G/T, and C/G and G/C were at similar levels (Figure 35).

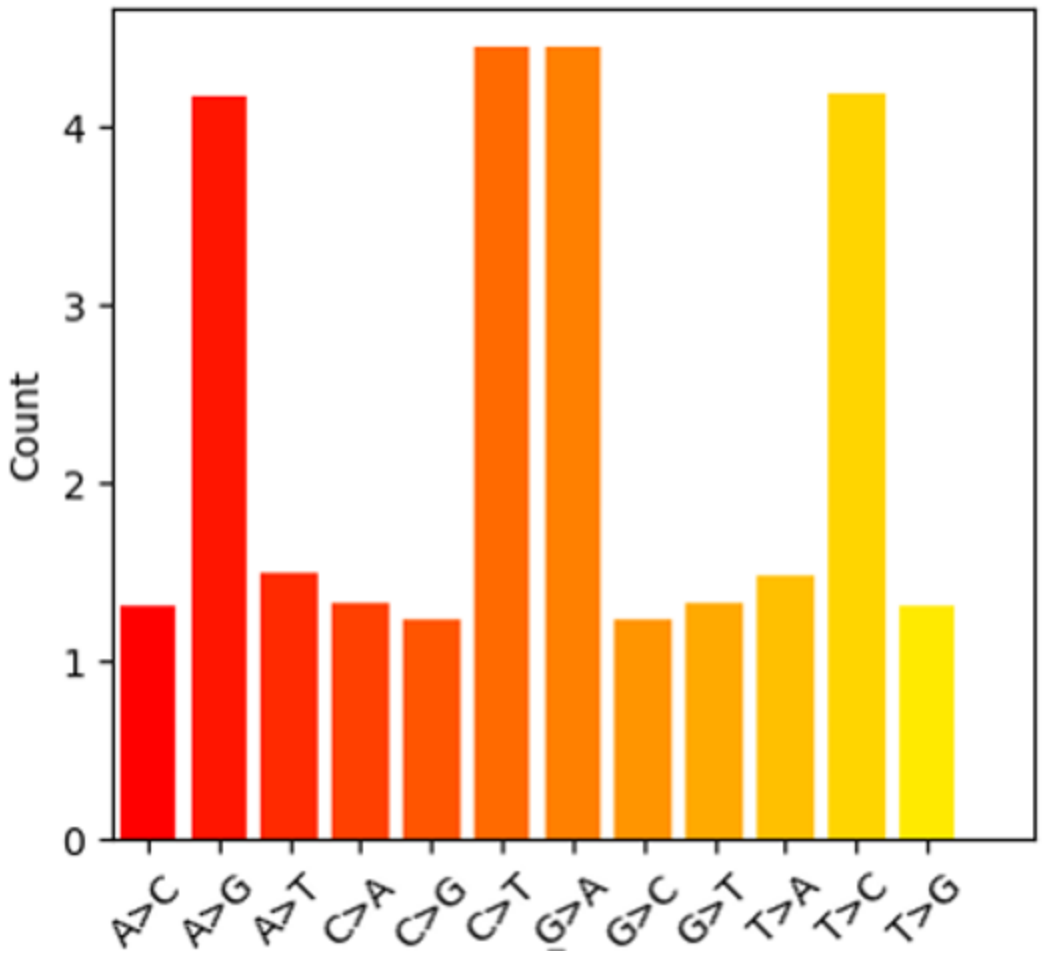

Figure 35: A substitution graph showing distribution of detected SNPs types (Transversions and Transitions) with each bar corresponding to the total counts. A>G, C>T, G>A and T>C are the SNPs with the highest counts.(transversions are more common compared to the transitions).

About 19.42 % of SNPs from the validation panel were A ↔ T and G ↔ C transitions representing 10.64 % and 8.78 % of the total SNPs, respectively. On the other hand a total of 80.58% of SNPs from the validation panel were A ↔ C, A ↔ G, C ↔ T and G↔ T transversions representing 9.38%, 30.89%, 30.91 % and 9.40% respectively (Table 10).

Table 10: A table representing the number of transitions or transversions.

S.No.	Substitution	No. of substitution	Substitution Type name
1.	A>T	148581	Transition

2.	C>G	122599	Transition
3.	G>C	122247	Transition
4.	T>A	147913	Transition
5.	A>C	130005	Transversion
6.	A>G	417100	Transversion
7.	C>A	131462	Transversion
8.	C>T	444180	Transversion
9.	G>A	444045	Transversion
10.	G>T	131825	Transversion
11.	T>C	417563	Transversion
12.	T>G	130073	Transversion

A total number of 2,787,593 SNPs sites were detected with a transition/transversion ratio of 1.62 (Table 11). The rate of mutation was not high. Fatal mutations occur when transition/transversion ratio is more than 2. From the total single nucleotide polymorphism counts the number of genes that were related to the *betta splendens.*

Table 11: Summary table representing the SNPs numbers representing the SNPs count and the transition/transversion (ts/tv) ratio.

	SNPs			indels		MNPs	others
Callset	n	ts/tv	(1st ALT)	n	frm*		
snps.	2,787,593	1.62	1.62	0	–	0	0
						* frameshift ratio: out/(out+in)	

	singletons (AC=1)			multiallelic	
Callset	SNPs	ts/tv	indels	sites	SNPs
snps.	100.0%	1.62	0.0%	0	0

CHAPTER FIVE

DISCUSSION

Fish is a crucial dietary component accounting for 17% of all the animal proteins consumed globally (Lancer and Bronnmann. 2022) and is a source of livelihood to over 600 million people (FAO, 2022). Fish is therefore an essential component in attaining SDG 2's goal of ending hunger and malnutrition, as well as tackling poverty and economic reliance in SDG 1. This is also espoused in priority area 1 and 2 of The Agenda 2063 goal 1 of the African Union which advocates for tackling poverty, inequality, and hunger.

Aquaculture has been on the upward trajectory and has become the main source of fish and fisheries products (FAO., 2020). Growing demand for fish and fisheries products has pushed fish farmers to produce more fish in smaller spaces, resulting in congested sub-optimal production settings that eventually harm fish growth performance. Fish genetics, feed quality and culture environment are key determinants of growth performance. While genetics and feeds have received considerable attention, little has been done with regards to the influence of fish culture water to fish growth performance. Fish raised in captivity develop in an environment that frequently becomes stressful. High stocking densities and ammonia are the two main chronic stressors present in many aquaculture facilities informing their choice in this study.

This study found that chronic stress resulted in depressed growth in juvenile *O. niloticus.* Fish raised in environments with greater ammonia concentrations exhibited slower weight gain (Figure3a) compared to the controls. Similarly, higher fish stoking density resulted in slower weight gain (Figure3b). On the other hand, fish raised in higher ammonia concentrations had significantly lower SGR values than the controls (Table 2). A similar

observation was also observed in the stocking density treatment (Table 3). The growth rates were positively correlated with the relative condition factor (K_n) in both cases. This is consistent with the assertion of Ighwela *et al*. (2011) that higher K_n values are indicative of fish wellness. High SGR and K_n values observed in the control fish indicates that the higher growth is associated with the fish wellness.

Chronic stress is the manifestation of the body's failure to return to normal homeostatic state after stressful episodes. Stress exposes an organism to negative effects including lowered growth, reduced reproduction, and weakened immunological function (Van Weerd and Komen, 1998). For greater productivity, disease prevention, and increased financial gain, aquaculture management practices must take stress into account and endeavor to minimize it. Glucocorticoid secretion is a typical endocrine reaction to stress that is produced in order to fuel the response to stress. In fish the major glucocorticoid produced during stress is cortisol and has been widely used as a stress indicator in fish (Ellis *et al*., 2011). One of the numerous functions of cortisol in the stress response is to provide the fish with enough energy to go through the perceived stressor and subsequently resume normal activity. Cortisol plays a major role in resource reallocation during stress. This reallocation lowers the reproductive axis, which is a prioritization of an individual's survival over the preservation of the species. Cortisol is essential for survival during stress and controls or supports several vital metabolic, immune, and homeostatic processes. For this reason, cortisol has found wide range of application in the study of stress in many vertebrates including fish.

Under stress, fish releases cortisol into the blood stream as a physiological stress response which causes the concentration of cortisol in the blood to rise. Plasma cortisol levels vary

with stress levels and are indicative of the status of the culture environment (Montero *et al.,* 1999). Values above basal means are indicative of stressful environment. The higher plasma cortisol concentrations witnessed in the experimental fish compared to the controls were due to the independent variables introduced in the experiment i.e. higher ammonia concentration and increased stocking densities and thus act as clear indicator of stress occurrence in the experimental fish.

Prolonged stress levels result in sustained high plasma cortisol levels which is eventually deposited in the scales of fish. Results of the current study indicate a positive correlation between the intensity of the stressor, the plasma cortisol concentration and the scale cortisol concentrations (Tables 4, 5, 8 and 9). Scale cortisol concentration can therefore be used as an indicator of stress levels the fish has been experiencing over a period of time. This finding concurs with Aerts *et al.* (2018) who worked on common carp and concluded that scale cortisol levels could be used as long-term stress indicator. A study by Samaras *et al., (*2021) found out that isolated scale under stimulation of ACTH is able to produce cortisol on their own. Thus, fish scales besides accumulating cortisol from blood may be carrying out de novo synthesis of the same. This coupled with the assertion that scales also participate in calcium homeostasis which would affect the cortisol storage in the scale may point to the fact that scale cortisol alone may not be sufficiently reliable in stress level determinations. This therefore points to the need for the use of a combination of markers during assessment of chronic stress in fish. From the results of the current study, an increase in fish plasma cortisol levels was accompanied by an increase in scale cortisol concentrations (Tables 4, 5 ,8 and 9)

Blood glucose is another important indicator of stress in fish. Chronic stress brings about hyperglycemia via enhancement of endogenous glucose synthesis and / or inhibition of glycogenesis in liver and skeletal muscles and which occurs by inhibition of glycogen synthase (Jensen *et al.,* 2011). According to Malini *et al*. (2018), when fish get bigger, hyperglycemia rises. In addition to fish size, environmental factors and the quality of the ecosystem also have an impact on hyperglycemia. The higher blood glucose levels recorded in the current study, is an indication of the increased levels of stress the fish were exposed to. Studies by (Makaras *et al.,* 2020) revealed that Plasma glucose concentrations exerts a positive correlation with increase in the level of stress. This is consistent with the findings of the current study (Table 6) where an increase in ammonia concentrations resulted in an increase in the mean plasma glucose concentrations. Similar observations were made for the stocking density stress (Table 7). Though they are the most commonly used signatures in stress level assessment, Cortisol and glucose suffer fluctuations lowering their accuracy (Goitao *et al.,* 2019). Thus their determination must be combined with other measurements, such as those of other stress hormones, Heat Shock Proteins (HSPS), blood-cell counts (preferably in long-term experiments), non-invasive techniques, and/or others, to provide a more comprehensive picture of a fish's stress status (Zhao *et al.,* 2015). Since stress affects both physiological and biochemical processes in fish, alteration of these processes can prove a useful addition to the methods of stress determination. Most of these processes affected by stress are regulated by the levels of circulating cortisol (Gaffey *et al.,* 2014).

The current study shows Mitogen Activated Protein Kinase (MAPK) pathway was significantly down regulated and may have a major influence on growth performance.

MAPK signaling pathway was significantly down regulated in ammonia treatment (Figure23). Six genes in this pathway were significantly enriched following ammonia treatment (Padj ≤0.05) i.e. DUSP1 (dual specific protein phosphatase 1), NHR38 (Nuclear Hormone Receptor), HSP 72 KDa Protein 1(heat shock protein), myelocytomatosis oncogene homologue (Mych), Growth arrest and DNA damage inducible alpha a (GADD 45aa) and mytogenic activated protein kinase kinase 4 (MAP2K4). Twenty-nine other genes in this pathway are also down regulated but not significantly (Padj. ≥0.05). Chronic stress emanating from different stressors has been shown to activate the MAPK signaling pathway (Eblen, 2018). Glucocorticoids have been reported to modulate the anti-inflammatory response via the inhibition of the MAPK signaling pathway. Cortisol has specifically been shown to suppress the phosphorylation of MAPK (Dong *et al.,* 2018), as well as the extracellular signal-regulated kinase (ERK1/2), p38MAPK and stress-activated protein kinase (JNK)/c-Jun N-terminal kinase. This suppression is mediated by DUSP-1(Wang *et al*., 2016) and forms the basis of the action of glucocorticoids cortisol being one of them. DUSP-1 is also referred to as (MAPK phosphatase (MKP) which mediates the de-phosphorylation of ERK (Solinas and Becattini, 2016). DUSP-1 has been shown to offer a negative feedback loop for MAPK / ERK pathway (Krysan and Colcombet, 2018). Kultz and Avila, (2001) demonstrated a reduction of MAPK activity in gills of euryhaline fishes subjected to osmotic stress. Results from the current study demonstrates enrichment of MKP. This enrichment results in reduction of the phosphorylated ERK which is the effector molecule of the classical MAPK pathway. Enrichment of MKP thus down regulates ERK signaling pathway inhibiting cell differentiation and proliferation hence depressing growth.

Witzel *et al*. (2012) reported that the blocking of the MAPK and ERK activation also activated apoptosis.

Results from the current study indicate that Nile tilapia exposed to chronic stress had significantly higher MKK4 levels consistent with the higher blood glucose level witnessed in stressed fish. High glucose levels has been shown to trigger JNK activation (Yung and Giacca, 2020). Phosphorylation of MKK4 leads to the activation of JNK. Prolonged activation of JNK activates apoptosis consistent with the depressed growth witnessed in this study. The coordination of gene transcription, protein synthesis, cell cycle control, apoptosis, and differentiation, orchestrated through MAPK regulation, assists in dealing with the effects of chronic stress and can therefore be viewed as an important adaptation towards stress management. Gadd45aa gene is down regulated in MAPK pathway as well as in the FOXO pathway. Forkhead box O (FOXO) is a member of a family of transcription factors that regulates the expression of genes in cellular physiological processes such apoptosis, cell-cycle regulation, glucose metabolism, oxidative stress resistance, and lifespan. FOXO transcription factors are regulated by its reversible acetylation and protein-protein interactions (Figure24). Acetylated FOXO are easily phosphorylated leading to FOXO induced gene expression. FOXO and especially FOXO1 plays a critical role in controlling the body's energy metabolism. Foxo regulates endogenous synthesis and absorption of glucose in peripheral tissues under the influence of insulin. Foxo1 also plays a critical role in the regulation of hepatic lipid metabolism (Sparks and Dong 2009). In addition foxo1 also plays an important role in the formation of both adipose tissue and skeletal muscle, two organs that are critical for maintenance of energy homeostasis. It has

been demonstrated in human beings that the acetylation of FOXO1 attenuates its transcriptional activity (Daitoku *et al*., 2004).

When nutrients are depleted, Foxo proteins, which function as the last effectors in insulin signaling pathways, induce gluconeogenesis in the liver, prevent adipose and myocyte differentiation, or switch the fuel that muscles use from glucose to lipids.

FOXO factors are fundamentally modified by phosphorylation and subsequent ubiquitination, which suppresses the expression of the target gene. The results from this study demonstrates a down-regulation of foxo signaling pathway in the ammonia treatment which in turn resulted in the down regulation of the expression of three genes including: Gadd45aa, BCL6a transcription repressor a and BCL6a transcription repressor b. Gadd45aa plays important immunological and apoptosis functions while BCL6aa and BCL6ab with pivotal roles in germinal centre formation and lymphocyte function, differentiation, and survival. On the other hand, stocking density treatment down-regulated two genes: BCL6aa and BCL6ab.

Foxo1a contributes to the growth of smooth muscle tissue, lymphatic vessel formation, and smooth muscle proliferation (Scallan *et al*., 2021). Foxo proteins have been demonstrated to play a role in the immune response in channel catfish after a bacterial infection (Gao *et al.*, 2019). Foxo has also been shown to induce apoptosis in drosophila under conditions of oxidative stress (Webb and Brunet 2014). In response to oxidative stress, it has been demonstrated that the down regulation of FOXO causes an increase in cell death in humans. Other than the MAPK signaling pathway and the FOXO pathway there were other pathways that were down regulated under ammonia stress including: the RNA degradation pathway, ErbB signaling pathway and the TGF Betta signaling pathway. These pathways

shares some genes that are downregulated, e.g. Mych is downregulated in ERB signaling pathway, TGF beta and MAPK signaling Pathway, while Map2k4, is downregulated in ERB and MAPK signaling pathways. These pathways are involved in immunity and muscle development. Chronic stress depresses immunity and growth in Nile tilapia.

Typically, a fish's stress reaction is characterized by an increase in muscular activity (Valentim *et al*., 2022). The cardiac muscle contraction, the calcium signaling pathway, and the adrenergic signaling in cardiomyocytes are important indicators of muscle function. Results of this study indicate that the pathways involved in these processes are enhanced in chronic stress scenarios employed in this experiment (Figure19). Muscle functioning requires the expenditure of energy in the form of ATP. Energy mobilization is significantly influenced by metabolic processes such as fatty acid breakdown, elongation, production of unsaturated fatty acids, and glycolysis/gluconeogenesis. The findings of this study document that these pathways are also enhanced in chronic stress. The reduction in growth performance of the chronically stressed fish compared to their non-stressed counterparts (controls) may be due to the divergence of energy from muscle development to muscular activities. Energy is redirected to organs, such as the brain and the skeletal muscles that react to stress by becoming more active. A study by Carneiro *et al*. (2022) reveals that Stressed fish have higher ventilation rates compared to unstressed controls and these rates have been used as a tool for assessing both alertness and stress levels in fish.

Results from the current study indicates that chronic stress up-regulated the cardiac muscle signaling pathway (Figure 22) and the adrenergic signaling in cardiomyocytes pathway (Figure19). In these two pathways, 15 common DEGs were significantly up-regulated. The cardiac muscle contraction pathway (Figure25) has an additional DEG (Cytochrome C

oxidase subunit 6B1which is key in electron transport chain) while the adrenergic signaling in cardiomyocytes has MAPK12a being up-regulated (Figure26). These up-regulated common DEGs are involved in facilitating muscle contraction. Some of the DEGS that were up-regulated in this pathway include ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 2a (ATP2a2a) and ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 1 (ATP2a1) which are involved in the transport of calcium into the lumen of sarcoplasmic reticulum (De Sena *et al*., 2021). Cardiac myosin light chain-1 (cmlc1) which enables calcium binding is also up-regulated. Calcium binding onto troponin brings about conformational changes on tropomyosin allowing the binding of myosin heads to actin to initiate muscle contraction (Valentim *et al*., 2022). In the current study, tropomyosin 2 (beta) and tropomyosin alpha-3 chain are up-regulated. These two proteins are involved in stabilization of the binding of actin and myosin regulating the tensing of the muscle fiber.

Cytochrome C oxidase subunit 6B1 and Na (+)/H (+) exchanger beta are also significantly enriched in this study. Cytochrome C oxidase subunit 6B1 is key in electron transport chain catalyzing the transfer of electrons to molecular O_2, driving Oxidative Phosphorylation. Na^+ /H^+ exchanger beta on the other hand, maintains intracellular pH (Bkaily and Jacques 2017) by exporting protons and importing Na^+. The cardiac muscle signaling pathway and the adrenergic signaling pathway complements each other in promoting muscular contraction (Bathe-Peters *et al*., 2021). The up-regulation of both cardiac muscle contraction and adrenergic signaling in cardiomyocytes is beneficial to the blood pumping process. Ekstrom *et al*. (2021) demonstrated that adrenergic activation enhances heart performance in acute thermal stress in various teleost.

Results of the current study indicates that these two pathways are up-regulated in chronic stress. Chronic stress has been shown to raise heart rate and blood pressure, exerting more strain on the heart's ability to pump blood to the body's various organs (Lopaschuk *et al*., 2021; Svendsen *et al*., 2021). The heart has a high energy requirement, in order to maintain its contractile function, it must continually produce large amounts of ATP. This ATP may be produced using a variety of substrates, such as fatty acids, carbohydrates, proteins, and ketones (Lopaschuk *et al*., 2021; Ripa *et al*., 2022). This process demands high amounts of oxygen resulting in the heart consuming more oxygen per unit weight than any other organ in the body (Ripa *et al*., 2022). According to Carneiro *et al*. (2022) stressed fish shows higher ventilation rates compared to unstressed controls.

Mitogen-activated protein kinase 12a (mapk12a) is thought to be involved in the calcium-independent control of smooth muscle contraction in differentiated smooth muscle. On the other hand, stocking density also up-regulated cyclic AMP (cAMP) responsive element binding protein 1 that functions as an activator of the phosphorylating enzyme, protein kinase A (PKA) (Kitagawa *et al*., 2016) and beta 2 adrenergic receptor that mediates smooth muscle relaxation (Tanaka *et al*., 2005). PKA is an important energy regulating enzyme which is responsive to the cellular levels of cAMP (Wang *et al*., 2018). The findings of this study have also demonstrated that chronic stress down regulates actin, alpha cardiac, the primary protein of the thin filament in cardiac sarcomeres, actin alpha I skeletal muscle a (acta1a) an ATP binding protein in skeletal muscle and cAMP responsive element modulator a (crema). Acta1a activates numerous other "myogenic genes," which are crucial for the development of muscles (Lee *et al*., 2017). Crema participates in a number of

intracellular activities, including neurogenesis, synaptic potentiation, differentiation, proliferation, and neuroplasticity (Cheng *et al*., 2013; Landeira *et al*., 2016). Taken together, the adrenergic signaling pathway promotes muscular contraction which is an energy expensive process and down regulates formation of muscle fiber compromising muscle growth. This is consistent with the findings of the growth performance analysis for the current study, which demonstrated that the experimental fish in the ammonia and the stocking density treatments both developed more slowly than the controls.

Alcohol dehydrogenase 1 (ADH1) which facilitates the inter conversion of alcohols and aldehydes or ketones with the reduction of nicotinamide adenine dinucleotide (NAD+) to NADH (Cheng *et al*., 2013), Carnitine palmitoyl transferase 1Ab (cpt1ab) which catalyzes the reversible exchange of acyl groups (which is derived from fatty acids) between coenzyme A and the acyl-CoA synthetase long chain family member 1a, which transforms free long chain fatty acids into fatty acyl-CoA esters and is involved in both lipid biosynthesis and fatty acid degradation (Chapman *et al*., 2014), were the three DEGs in the fatty acid degradation pathway (Figure27) that were up-regulated by increased ammonia levels. Chronic stress also up-regulated the biosynthesis of unsaturated fatty acids and fatty acid elongation pathways. The DEGS that were up-regulated in these pathways are; Acyl-coenzyme A thioesterase 4 (ACOT 4), acyl-coenzyme A thioesterase 1 (ACOT 1), ELOVL family member 6 (Elovl6l), elongation of long chain fatty acids like genes were the significant DEGs that were up-regulated in this pathway and are involved in the regulation of intracellular levels of CoA esters. Carnitine palmitoyl transferase 1Ab and alcohol dehydrogenase 1 are also significantly up-regulated. ACOT4 plays a crucial role in both fatty acid and peroxisomal lipid metabolism. ACOT 4 is a paralog of ACOT1.

Biosynthesis of unsaturated fatty acids, fatty acid elongation and fatty acid degradation DEGs are significantly up-regulated in stress. The levels of acetyl CoA determines which processes biosynthetic or degradation will occur. Acetyl-CoA carboxylase the only controlled and the primary enzyme in fatty acid synthesis is phosphorylated / dephosphorylated by both AMP-activated protein kinase (AMPK) and Protein Kinase A. De-phosphorylation of Acetyl-CoA carboxylase activates it and promotes fatty acid biosynthesis while its phosphorylation promotes fatty acid degradation. AMP Kinase is an energy status sensor that controls cellular and overall energy balance in the body (Lee *et al*., 2018). AMPK maintains the energy balance by regulating metabolic processes such as mitochondrial synthesis, autophagy, fatty acid oxidation, glucose and lipid transport, and oxidative metabolism while decreasing ATP-consuming processes like glucose, proteins, fatty acids and cholesterol synthesis (Rotte *et al*., 2010). Results from the current study indicate that under chronic stress, AMPK is up-regulated thus promoting fatty acid degradation. Chronic stress was also seen to down regulate gamma enolase, a key enzyme in glycolysis and gluconeogenesis. Gamma enolase is particularly active in cells undergoing increased aerobic glycolysis (Vizin *et al*., 2015). In this study, gamma enolase was significantly down regulated pointing to the fact that glycolysis was down regulated hence fatty acid degradation may be the principal source of metabolic energy during stress.

Results of the current study also indicated a significant increase in blood glucose level in stressed fish compared to the controls. The decreased utilization of glucose may therefore be associated with this accumulation of glucose in the blood. Several other studies (Zheng *et al*., 2018; Mulei *et al*., 2021; Chau *et al*., 2022) have also associated chronic stress with hyper glycaemia in fish. Activation of AMPK has also been shown to influence the

functioning of Na^+ / H^+ exchanger promoting the export of H+ out of the cell in exchange of Na+ (Cao *et al*., 2020). In the current study both AMPK and Na+ /H+ exchanger were significantly up-regulated. This predisposes the cell to degradative pathways rather than anabolic pathways hence growth is compromised. Growth performance analysis conducted for the current study, revealed that the experimental fish in the ammonia and stocking density treatments both grew more slowly than the unstressed controls.

The significant up-regulated DEGs annotated to the apelin signaling pathway include the myosin light chain kinase family, member 4a (mylk4a), myosin light chain 2b regulatory cardiac slow (myl2b), and the early growth response 1 (egr1) a regulator of cell proliferation and apoptosis. Myosin regulatory light chains are phosphorylated by the enzymes mylk4a and myl2b to help myosin connect with actin filaments and induce contractile activity (Yarahmadi *et al*., 2016). The up regulation mylk4a and myl2b is evidence of heightened muscle activity which in turn leads to higher energy consumption. Myosin, light chain 13(myl13), cardiac myosin light chain-1(cmlc1) and ryanodine receptor 1 were up-regulated in this pathway in ammonia treatment and are actively involved in muscle activity. Na^+/H^+ exchanger beta helps to maintain the correct pH for muscular activity (Masri *et al*., 2005; Fearnley *et al*., 2011; Cho *et al*., 2017). On the other hand, cadherin-1 a gene involved in cell- cell adhesion and is of fundamental importance in the development and maintenance of tissues was down regulated in the stocking density treatment. Taken together higher consumption of energy and reduced cell-cell adhesion results in depressed growth performance in stressed fish. The apelin signaling pathway was up-regulated in chronic stress. Numerous physiological processes and pathological conditions, such as cardiovascular disease, angiogenesis, energy metabolism, and fluid

balance, are influenced by the apelin/APJ system (Chapman *et al*., 2014). Due to its involvement in muscle movement, cooperatively with cardiac signaling pathway and adrenergic signaling pathway, it suffices to infer that chronic stress increases muscular activity and thus energy consumption at the expense of growth. This is in concurrence to the growth performance results in the current study which shows that the experimental fish in both ammonia and stocking density treatments had a lower growth rate as compared to the controls. In a previous study, Volkhoff *et al*. (2009), demonstrated an increase in apelin with fasting (stress) in goldfish (*Carassius auratus*). Apelin signaling has also been shown to affect the intake of food and water, as well as the central and peripheral control of the cardiovascular system (Masri *et al*., 2005).

Calcium signaling pathway was also significantly up-regulated in both ammonia and stocking density treatment. The function of cardiac myocytes is significantly regulated by Ca^{2+}. Calcium is primarily the connection between electrical impulses that are sent throughout the heart and myocyte contraction to move blood (Fearnley *et al*., 2011). During contraction and relaxation, Ca2+ in the cytosol activates and deactivates a variety of contractile proteins (Lee *et al*., 2010)**.** DEGs like **5**-hydroxytryptamine receptor 7, myosin light chain kinase family member 4a (mylk4a), fibroblast growth factor receptor 4 (fgfr4), adenosine A2a receptor a (adora2aa), immunoglobulin mu heavy chain-like and beta-2 adrenergic receptor were up-regulated in the calcium signaling pathway in the stocking density treatment and are involved in muscle activity and signal transduction.

Ammonia treatment up-regulated seven DEGs including; solute carrier family 25 member 5 (slc25a5), troponin c slow skeletal and cardiac muscles, ATPase Sarcoplasmic / Endoplasmic reticulum Ca^{2+} transporting 1 (ATP2a1), Ryanodine Receptor 1, troponin c

type 1a (slow) (tnnc1a) and 5-hydroxytryptamine receptor 7. These DEGs are important components of muscle contraction and relaxation cascade. Altogether, the pathways discussed above involved in muscle activity and energy mobilization. The effect of increased muscle activity and thus energy consumption accompanying chronic stress attenuates growth as evidenced in this study. A previous study by (Lee *et al*., 2010) reported up-regulation of Ca2+signaling pathway and troponins and glycolytic genes in superior performing hybrid grouper. These were associated with enhanced muscle activity and growth. In the current study enhanced muscle activity in stress correlates to depressed growth. Several studies on growth performance have focused on the insulin like growth factors, their receptors, growth hormones and their receptors, my statin and somatolactin (Lee *et al*., 2010; Zhou *et al*., 2010; Li *et al*., 2013; Yarahmadi *et al*., 2016;). Most of these studies involved acute stress. In the current study, these genes were not significantly up-regulated. It remains to be found out whether they could have fallen back to basal levels with time.

Unlike chronic stress, acute stress has received tremendous research interest due to its calamitous effects. Even though chronic stress may not result in fish kills, the economic losses arising from lost productivity and mitigation measures are enormous. Furthermore, the deterioration of fish flesh quality after slaughter in terms of colour, flavour, firmness, weight loss / water holding capacity and perishability have been documented (Huang *et al*., 2023). Many studies on chronic stress have focused on stocking density, temperature and anoxia. Few studies have attempted to explain the molecular basis of depressed growth in fish under chronic ammonia exposure. This is the first report ever attempting to decipher how sub lethal concentrations of ammonia affect *O. niloticus* growth. Results of the current

study contributes to the number of molecular mechanisms explaining growth depression associated with chronic stress.

Ammonia treatment up regulates 16 DEGs in the cardiac muscle signaling pathway with three of these DEGs being also up regulated in the stocking density treatment. Three DEGs in this pathway were significantly down regulated in stocking density treatment while only one DEG was down regulated in ammonia treatment. In the adrenergic signaling pathway, ammonia treatment up regulated 16 DEGs and down regulated 2 DEGs while the stocking density treatment only up regulated 5 DEGs and down regulated 3 DEGs in the same pathway. Ammonia treatment up regulates muscle activity related genes as compared to stocking density treatment.

Results from this study indicate that stocking density up regulated the intestinal immune pathway. MAMU histocompatibility antigen, Histocompatibility class II antigen and immunoglobulin Mu heavy chain were significantly up regulated in this pathway. On the other hand, only immunoglobulin mu heavy chain was up regulated in ammonia treatment. Eight DEGs were significantly up regulated in the phagosome pathway and one was significantly down regulated in the stocking density treatment while four DEGs were significantly up regulated and one down regulated in ammonia treatment. Class I histocompatibility antigen and immunoglobulin MU heavy chain like genes were significantly up regulated in both ammonia and stocking density treatments. Stocking density stress up regulated several common genes in the intestinal immune, phagosome and cell adhesion pathways (Figure21). These genes include MAMU histocompatibility antigen, histocompatibility class 2 antigen, Class II histocompatibility antigen and

immunoglobulin MU heavy chain like which are involved in immunity. Stocking density stress downregulates immunity in Nile tilapia.

Interleukin 8 (IL-8) was also significantly down regulated in this study. Similar findings were obtained when *O. mykiss* was subjected to increased stocking densities (Yarahmadi *et al*., 2016). In this study ammonia down regulated IL-8 though not significantly. A study by Liu *et al*. (2020) showed that acute ammonia stress up regulates IL-8 in Pacific white shrimp (*Litopenaeus vannamei).*

Stocking density down regulated Super oxide dismutase 1 (SOD1) and catalase (CAT) while it up regulates SOD2 and SOD3 but not significantly. In *Labeo lohita*, SOD and CAT were up regulated when subjected to increased stocking density. In Chinese sturgeon SOD and IL-8 were significantly down regulated by increasing stocking density (Long *et al.,* 2019). In ammonia treatment, results from this study indicate that SOD 1, 2 and 3 were down regulated though not significantly. Catalase was up regulated in this treatment but not significantly. In Pacific white shrimp, ammonia has been shown to down regulate SOD and catalase (Liang *et al*., 2016). In puffer fish (*Takifugu obscurus)* ammonia up regulated catalase and SOD (Cheng *et al*., 2015). These observations amplifies the findings of (Fardell *et al*, 2021) that stress reaction varies according to type and intensity of stressor as well as the species involved and the stage of development.

The findings of the current study indicates that stocking density up regulates immune related genes. This is in contrast to the findings of Shourbela *et al*. (2021) that at low stocking densities, tilapia immunity was impaired. The study by Shourbela *et al*. (2021) however, showed that increasing stocking density enhanced the expression of innate

immune genes in Atlantic salmon. The transcriptional response to chronic stress and the changes in gene expression and signaling pathways in the current study suggest that the aforementioned DEGs and pathways play a significant role in the mechanism behind Nile tilapia's enhanced tolerance to chronic stress.

In addition to ammonia and stocking density, the cultural environment of fish also contains other stresses. Besides the aforementioned genes, fish therefore possess genes that enable them to adapt to the ensuing stress from these stressors. To identify these genes that confer these beneficial traits a pan genome analysis was carried out. The pan genome comparison of the whole genome sequence scripts revealed the introgression of genes into the experimental fish experimental fish from *O. aureus* and *Betta splendens* (Figure29). The phylogenetic tree developed from the shared SNP markers showed significant separation between the fish species. The tree showed *letimeria chalumnae* and *Prionailurus viverrinus* as the most distant and had diverged in times together with *Danio rerio,* followed by *Betta splendens.* There were two different clusters of *Oreochromis* that diverged together in times: *O. aureus* and *O. niloticus*. The experimental fish is a close relative of *O. niloticus* as they clustered together in the same clade (Figure30).

The pan genome analysis revealed a total of 8,045 core genes and 19,532 variable genes were identified among four species that showed a close genetic relationship (Figure 31) Experimental sample and *Oreochromis aureus* had 59 common unique genes. Since Experimental sample is a strain of *Oreochromis niloticus,* the 59 genes probably have been introgressed from *Oreochromis aureus.* On the other hand, 18 genes were common between *Betta splendens* and Experimental sample that may probably have arisen in this strain through hybridization between *O. niloticus* and *O. aureus.* Among the 18 genes we

identified four introgressed genes that conferred beneficial traits to our Experimental sample including the S100 calcium binding protein B (S100B) that reduces *il6* production in malignant melanoma via inhibition of RSK cellular signalling, the triple functional domain protein-like that is involved in coordinating actin remodelling, which is necessary for cell migration and growth, Peroxiredoxin-like 2A, an adipocyte-derived PAMM that may suppress macrophage activation by inhibiting MAPK signalling pathway and the coxsackievirus and adenovirus receptor homolog that is thought to regulate the cytoskeleton through interactions with actin and microtubules in fish. A total of 96 genes were common to *Betta splendens*, *Oreochromis aureus* and experimental sample but were absent in *O. niloticus*. By contrast, the experimental fish gained 4179 genes while losing 6118 genes overall compared to *O. niloticus*. It is plausible to hypothesize that the introgressed genes are the basis of the beneficial phenotypes exhibited by experimental sample in terms of resilience to stress, immunity, and faster growth in comparison to *O. niloticus* (Figure31). Among the unique genes found in experimental sample in this study and absent in *O. niloticus* in this study were classified according to the functions they augmented either immunity, stress resistance or growth making experimental sample better adapted to the local environmental conditions. Some of the selected genes exhibiting substantial effect on immunity include: Peroxiredoxin, Macrophage mannose receptor 1-like and Zinc finger MYM type protein 4-like partial while stress reactive genes included Palladin like gene, large proline rich protein BAG6 like and growth-related genes such as Nuclear factor 1-x-type like, pigment epithelium derived factor and cathepsin L like.

O'Leary *et al* (2014) demonstrated that Peroxiredoxin-1-like can directly interact with other proteins, which may have an impact on other cellular processes including apoptosis,

iron metabolism, proliferation, and the growth and operation of tissues, organs and systems. In pathogen infection, as well as in defence against cell death, tissue healing after injury, and tumour growth, they may function as inflammatory modulators. According to Krata *et al*. (2021) peroxiredoxins modulates oxidative stress and can also be used as indicators of oxidative stress in humans. Peroxiredoxins are crucial regulators of oxidative stress because they effectively reduce peroxides levels thereby regulating the peroxidases signalling. Peroxiredoxins accounts for around 90% of the decline of peroxidases activity in the body (Perkins *et al*., 2015). Peroxiredoxin has also been demonstrated to act as a chaperon for apurinic endonuclease (APE 1) (Nassour 2016) which is essential for the activity of interleukin 8 (il-8) and Nuclear factor kappa B (nf-ϰB) (O'Leary *et al.*, 2014). Il-8 and nf-ϰB plays an important role in ammonia and heat stress resistance in fish (Esam *et al* 2022). Therefore, peroxiredoxin is an important component in stress management as it is directly involved in oxidative, ammonia and thermal stress management.

Paladin-like protein is an important protein in stress response. It attracts VASP to these sites to aid in the formation of dorsal stress fibres (Price and Brindle, 2000). When stress fibres started to form in Rcho-1 cells of adult mice, Palladin expression was seen to rise. Palladin is widely distributed in developing tissues, which raises the possibility that this protein plays a unique role in the organization of the actin cytoskeleton in cells that are differentiating structurally and functionally (Parast *et al*., 2000). Large proline-rich protein BAG6-like endoplasmic reticulum stress-induced pre-emptive quality control BAG6 is also involved in endoplasmic reticulum stress-induced pre-emptive quality control, a process that reroutes freshly produced proteins to the cytosol for proteasomal destruction while selectively attenuating their translocation into the endoplasmic reticulum (Kadowaki

et al., 2018). Misfolded proteins are broken down when they are moved from the endoplasmic reticulum and directed to the cytoplasmic proteasomes for destruction. This study revealed our experimental fish had the BAG6 gene introgressed into its genome implying a superior stress management as compared to its nilotic parents. It is plausible to hypothesize that the introgression of the BAG6 gene into tilapia and related cichlids, would improve their stress resistance and thus improve productivity. Cathepsin L, a cysteine protease belonging to the papain superfamily, is essential for carrying out typical cellular processes such as general protein turnover, antigen processing, and bone remodelling essential to the architecture and function of the heart plays a crucial part in the morphogenesis and cycling of hair follicles as well as the differentiation of the epidermis. Endopeptidase activity, formed as a pro-enzyme, in lysomes. A papain-like lysosomal enzyme called cathepsin L (CTS-L) breaks down endocytosed proteins to produce immunogenic antigens for adaptive immunity (Zhu *et al*., 2022). According to Chen *et al.*, (2020), Cathepsin has also been reported to be linked with the presentation and processing of antigens, as well as the control of immune responses in turbot fish. The endosomal/lysosomal system's nonspecific bulk proteolysis, which breaks down both intracellular and extracellular proteins, is predominantly carried out by cathepsins, which are primarily intracellular enzymes. However, because of the restricted proteolysis processing, cathepsins play a role in the production of immune modulators. As a result, cathepsin L is a crucial gene that affects how the innate immune system responds.

MMR1-like enables macrophages to phagocytose and endocytose glycoproteins binds polysaccharide chains that are both sulphated and unsulfated. MMR1-like interacts with the glycoproteins and glycolipids that are present on the surface of bacteria, fungi, and

viruses that cause disease. Studies on zebra fish show that the MMR exhibits expression in every tissue investigated and shares highly conserved structures with MMRs from other species (Zheng *et al*. 2015). The fact that MMR is expressed in the kidney and spleen indicates that it is involved in the immunological responses to infection. Nuclear factor 1 x-type- like *(NFix)* recognizes and binds the palindromic sequence and has been shown to play an important role during development especially in stem cell differentiation, maturation and self-renewal (Haris *et al.*, 2015).

Yu *et al* (2022) demonstrated a mixed ancestry for the Sukumandi strain which had genes from Nile tilapia (*O. niloticus)* with approximately 0.36% of the genome having been derived from the blue tilapia (*O. aureus*). These shared genes may have arisen from the interbreeding between *O. niloticus* and *O. aureus*. About 0.11% of the genome was derived from *Betta splendens*. Hybridization of female *O. niloticus* and male *O. aureus* have been used to obtain predominantly male offsprings that are desirable for aquaculture. This aspect of interbreeding between *O. niloticus* and *o. aureus* also happened in the wild environments. The experimental sample has accumulated genes from other fish species making it more adaptive to environmental stressors as compared to *O. niloticus.*

To assess the quality of the SNPs calls, the ts/tv ratio was calculated.

Data provided indicates the existence of genetic admixture ancestry in experimental sample. From the results of the current study, 155 genes are shared between experimental sample, betta splendens and *O. aureus,* which are absent in *O. niloticus*. This clearly demonstrates that the alleles have been introgressed into *O. niloticus* from *O. aureus* and *Betta splendens*. In the wild *O. niloticus, B. splendens* and *O. aureus* live in the same environment. There is tangible evidence that these fish can interbreed to produce an

offspring of mixed ancestry and in the process acquire and transmit genes that are not traditionally found in them. With this background it is plausible to come up with breeding programmes that target genes conferring advantageous traits to the particular fish. Desirable traits such as fast growth, resistance to disease and parasite, tolerance to high salinity, high stocking densities and high levels of ammonia are important in the improvement of the aquaculture industry. There are established methods of delivering selected genes such as Tol2 system and CRISPR-Cas9 (Li *et al*., 2016). Tol2 transgenesis system that is based on Tol2 transposon, a mobile genetic element that has been found to function in zebrafish and other fish species (Keng *et al*., 2009). The CRISPR-Cas9 on the other hand is an RNA directed endonuclease that can generate double stranded breaks in the genome. It can also be used for whole genome genetic screens.

Betta splendes and *Danio rerio* species have a better salinity tolerance compared to *O. niloticus* and *O. aureus*. *Betta splendens* has a solute carrier family 25-member 24(Slc25a24) gene, an ATP Mg/Pi carrier, which is involved in the control of energy metabolism which enable it to cope with the salinity stress (Yu *et al*., 2022). This gene is absent in *O. aureus* and *O. niloticus* but is present in *Danio rerio* species. On the other hand, *Danio rerio* also has Solute carrier family 12 member 10, tandem duplicate 1 gene which plays a key role in sodium, potassium and chloride ions homeostasis. The protein expressed from this gene enables sodium potassium symporter activity. It is the authors considered opinion that the introgression of these two genes into the genome of *O. niloticus* could improve its salinity tolerance and allow it to be adaptable to saline water environments. In result, this will expand the aquaculture scope from not only fresh water bodies but also to marine and brackish water environments.

Danio rerio is more tolerant to hypoxic conditions relative to *O. niloticus*, *O. aureus* and betta *splendins*. This can be associated with the presence of genes such as ATP binding cassette transporter a1 (*Abca1),* B-cell lymphoma 2a *(bcl2a)* and (*mylk3)*. *Abca1* is also present in *O. aureus* and has been shown to increase hypoxia tolerance by increasing cholesterol efflux from peripheral cells, particularly foam cells in atherosclerotic plaques (Bogomolova *et al.,* 2019). *Abca1* helps in clearing cholesterol in the atherosclerotic plaques and loading it to the *ApoA-1* which transports it to the liver. This clearance reduces the thickness of the arterial walls allowing for oxygen diffusion (Linton *et al.,* 2000). *Bcl2a* prevents cell death by blocking oxidative stress induced apoptosis (Sunsnow *et al.,* 2009). On the other hand, *mylk3* gene has been shown to be a key gene in the regulation of cardiac myocyte contraction. This enables the heart to contract faster and more intensely to deliver more blood and thus oxygen to the body cells (Tobita *et al.,* 2017). The evidence of the beneficial traits conferred by these genes and their absence in *O.aureus* and *O. niloticus* provide a plausible ground to aver that their introgression would improve hypoxia tolerance of *O. niloticus.* This will help in overcoming the challenge of hypoxia stress which is a main limiting factor in tilapia aquaculture intensification.

Taken together introgression of salinity and hypoxia tolerance genes into *O. niloticus* would benefit the aquaculture industry by providing Strains that would not only survive in these stressful conditions but also yield highly. Breeding for salinity tolerance and hypoxia tolerance would be a suitable area of interest that will enable aqua culturists to come up with parental generations that contains these genes and thus can pass them down naturally to the next generations. Breeding for a specific trait would be a faster and more accurate method compared to pedigree-based method (Vallejo *et al.,* 2017; Yoshida *et al.,* 2018).

Some of these prospective genes may be employed as candidate gene indicators of chronic stress tolerance in future breeding programs.

The results of the current study increase the number of molecular markers available as tools for selective breeding in fish. Fish breeding for stress tolerance is an important tool towards increasing fish production through developing new better performing stress tolerant fish species. This will lower cost of production and minimize the occurrence of diseases and mortality in cultured fish. With the ever-increasing world population, the demand for fish and fish products continue to increase. Coupled with the dwindling catches from the wild, there is need to produce more fish in less and less area. The strain occasioned will most likely increase occurrence of stress in the cultured fish with the far-reaching consequences of depressed growth and increased mortality. As a result, this will dent the effort towards achieving freedom from hunger and malnutrition as espoused in SDG 2 as well as sink more farmers in to poverty and economic dependence contrary to SDG 1. It is therefore important to formulate policies that promote fish welfare to guard against occurrence of chronic stress in fish such as water pollution and global warming.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.0 Conclusions

1. This study demonstrates a variation in the level of growth performance of cultured Nile tilapia with the cultured fish showing depressed growth after being subjected to different ammonia concentrations and stocking densities.
2. This study demonstrates that cultured Nile tilapia suffers stress when subjected to concentrations of un-ionized ammonia above 1.2 mg/ L and stocking densities above 55 fish per 500 L tank.
3. This study demonstrates an association between differentially expressed genes in Nile tilapia cultured at different ammonia concentrations and stocking densities.
4. This study demonstrates a genetic sequence variation between the cultured Nile tilapia and the *Oreochromis niloticus* in the NCBI database.

6.1 Recommendations

1. For best performance Nile tilapia should be cultured in waters with un-ionised ammonia levels not exceeding 1.2 mg/L.
2. Low stocking densities below 55 fish per tank of 500 L be adopted for better productivity of cultured Nile tilapia.
3. Further studies to enable the selection of best candidate genes for selected breeding programs for stress tolerance be conducted from among the genes identified.
4. Pan genome analysis should be carried out to identify beneficial genes that could be introgressed to obtain a better stress tolerant fish strain.

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APPENDICES

APPENDIX 1: PROPOSAL APPROVAL

MASINDE MULIRO UNIVERSITY OF SCIENCE AND TECHNOLOGY (MMUST)

Tel: 0702597360/61:
0733120020/22
E-mail: directordps@mmust.ac.ke
Website: www.mmust.ac.ke

P.O Box 190
50100 Kakamega
KENYA

Directorate of Postgraduate Studies

Ref: MMU/COR: 509079

18th January 2022

John Gitau Mwaura
SBB/H/01-53968/2019
P.O. Box 190-50100
KAKAMEGA

Dear Mr. Mwaura,

RE: APPROVAL OF PROPOSAL

I am pleased to inform you that the Directorate of Postgraduate Studies has considered and approved your Ph.D. proposal entitled: ***"Growth Performance and Transcriptome Analysis of Deferentially Expressed Genes in Tilapia (Oreochromis niloticus) Subjected to Chronic Stress"*** and appointed the following as supervisors:

1. Dr. Patrick Okoth - Department of Biological Sciences - MMUST
2. Dr. Phillip Oguttu - Department of Biological Sciences - MMUST

You are required to submit through your supervisor(s) progress reports every three months to the Director of Postgraduate Studies. Such reports should be copied to the following: Chairman, School of Natural Sciences and Technology Graduate Studies Committee and Chairman, Department of Biological Sciences. Kindly adhere to research ethics consideration in conducting research.

It is the policy and regulations of the University that you observe a deadline of three years from the date of registration to complete your Ph.D. thesis. Do not hesitate to consult this office in case of any problem encountered in the course of your work.

We wish you the best in your research and hope the study will make original contribution to knowledge.

Yours Sincerely,

Prof. Stephen O. Odeberi, PhD, FISEP
DIRECTOR, DIRECTORATE OF POSTGRADUATE STUDIES

APPENDIX 2: MMUST INSTITUTIONAL SCIENTIFIC ETHICS REVIEW COMMITTEE (ISERC) CERTIFICATE

MASINDE MULIRO UNIVERSITY OF SCIENCE AND TECHNOLOGY

Tel: 056-31375
Fax: 056-30153
E-mail: ierc@mmust.ac.ke
Website: www.mmust.ac.ke

P. O. Box 190,
50100.
Kakamega,
KENYA

Institutional Ethics and Review Committee (IERC)

REF: MMU/COR: 403012 Vol 5 (01)

Date: December 16th, 2021

To: John Gitau Mwaura

Dear Sir,

RE: GROWTH PERFORMANCEAND TRANSCRIPTOME ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES IN NILE TILAPIA (*Oreochromis niloticus*) SUBJECTED TO CHRONIC STRESS.

This is to inform you that ***Masinde Muliro University of Science and Technology Institutional Ethics and Review Committee (MMUST-IERC)*** has reviewed and approved your above research proposal. Your application approval number is **MMUST/IERC/041/2021.**The approval period is ***December 16th, 2021-December 16th, 2022.***

This approval is subject to compliance with the following requirements;

i. Only approved documents including informed consents, study instruments, MTA will be used.
ii. All changes including (amendments, deviations, and violations) are submitted for review and approval by ***MMUST-IERC.***
iii. Death and life threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to ***MMUST-IERC*** within 72 hours of notification
iv. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to ***MMUST-IERC*** within 72 hours
v. Clearance for export of biological specimens must be obtained from relevant institutions.
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
vii. Submission of an executive summary report within 90 days upon completion of the study to ***MMUST-IERC.***

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://research-portal.nacosti.go.ke and also obtain other clearances needed.

Yours Sincerely,

Prof. Gordon Nguka
Chairperson, Institutional Ethics and Review Committee

Copy to:
- The Secretary, National Bio-Ethics Committee
- Vice Chancellor
- DVC (PR&I)

APPENDIX 3: RESEARCH CERTIFICATE FROM NACOSTI

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **414600**

Date of Issue: **23/May/2020**

RESEARCH LICENSE

This is to Certify that Mr.. John Gitau Mwaura of Masinde Muliro University of Science and Technology has been licensed to conduct research in Kakamega on the topic: GROWTH PERFORMANCE AND WHOLE TRANSCRIPTOME ANALYSIS OF DIFFERENTIALY EXPRESSED GENES IN CULTURED NILE TILAPIA (Oreochromis niloticus) SUBJECTED TO CHRONIC STRESS for the period ending 23/May/2021

License No: **NACOSTI/P/20/5012**

414600

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

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APPENDIX 4: GENES / FUNCTIONS AFFECTED BY STRESS IN FISH

	Gene	**Species**	**Ref.**
1.	Heat shock protein 70	*O. mykiss*	Wang *et al*., 2008, Zahediet *et al*.,2019
2.	IL-1β (interleukin -1 beta	*O. mykiss*	Wang *et al*., 2008, Shepherd *et al*., 2018
3.	SOCS-1 and SOCS-2 (Supressor of Cytokines 1 and 2)	*O. mykiss*	Shepherd *et al*., 2018
4.	IGF -1(Insulin like Growth factor)	*O. mykiss*	Shepherd *et al*., 2018, Zahediet *et al*., 2019
5.	GR (Glucocorticoid Receptor)	*O. mykiss*	Shepherd *et al*., 2018
6.	Prolactin		Pavlidis *et al*., 2015
7.	sst1 (somatostatin 1)	*O. niloticus*	Rodriguez-Barreto *et al*., 2019
8.	Fosab,	*O. niloticus*	Rodriguez-Barreto *et al*., 2019
9.	Pomca	*O. niloticus*	Rodriguez-Barreto *et al*., 2019
10	Grp (gastrin releasing peptide)	*O. niloticus*	(Balment, Lu, Weybourne, & Warne, 2006
11	Hypocretin/ orexin neuropeptide precursor (hcrt)	*O. niloticus*	Balment, Lu, Weybourne, & Warne, 2006
12	Oxytocin (oxt)	*O. niloticus*	Balment,Lu , Weybourne, & Warne, 2006
13	Vasopressin (Vp)		Bernier, 2006
14	Thyrotropin- releasing hormone (trth)		Jezova, *et al*.,1995
15	growth hormone-releasing hormone receptor (ghrhrl)		Pavlidis *et al*., 2011).
16	Parathyroid hormone 1 receptor (pth1ra)		Pavlidis *et al*., 2015).
17	MST-1α (Myostatin – 1 α)	*O. mykiss*	Zahediet *et al*., 2019
18	MHC (Myosin Heavy Chain)	*O. mykiss*	Zahediet *et al*., 2019
19	FST (Follistatin)	*O. mykiss*	Zahediet *et al*., 2019
20	(CAT)Catlase	*O. niloticus*	Goes *et al* ., 2019
21	(GPx) Glutathione peroxidase	*O. niloticus*	Goes *et al* ., 2019
22	(HSP 70) Heat Shock Protein	*O. niloticus*	Goes *et al* ., 2019
23	(RyR1) Ryanodine Receptor 1	*O. niloticus*	Goes *et al* ., 2015
24	(RyR3) Ryanodine Receptor 3	*O. niloticus*	Goes *et al* ., 2015

APPENDIX 5: MANUSCRIPT 1

genes

MDPI

Article

Whole Transcriptome Analysis of Differentially Expressed Genes in Cultured Nile Tilapia (*O. niloticus*) Subjected to Chronic Stress Reveals Signaling Pathways Associated with Depressed Growth

John Gitau Mwaura [1,2], Clabe Wekesa [1], Philip A. Ogutu [1] and Patrick Okoth [1,*]

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* Correspondence: okothpatrick@mmust.ac.ke

Abstract: Chronic stress is a serious threat to aquaculture as it lowers fish growth performance and compromises fish welfare. The exact mechanism by which growth is retarded is, however, not clearly understood. This study sought to elucidate the gene expression profiles associated with chronic stress in cultured Nile tilapia (*Oreochromis niloticus*) reared for 70 days at different ammonia concentrations and stocking densities. Fish in the treatment groups showed negative growth, while the controls showed positive allometric growth. The specific condition factor (K_{sp}) ranged from 1.17 for the controls to 0.93 for the ammonia and 0.91 for the stocking density treatments. RNA was extracted from muscle tissue using TRIzol followed by library construction and Illumina sequencing. Comparative transcriptome analysis revealed 209 differentially expressed genes (DEGs) (156 up- and 53 down-regulated) in the ammonia and 252 DEGs (175 up- and 77 down-regulated) in the stocking density treatment. In both treatments, 24 and 17 common DEGs were up- and down-regulated, respectively. DEGs were significantly enriched in six pathways associated with muscle activity, energy mobilization and immunity. The heightened muscular activity consumes energy which would otherwise have been utilized for growth. These results bring to fore the molecular mechanisms underlying chronic stress' suppression of growth in cultured Nile tilapia.

Keywords: chronic stress; differentially expressed genes; ammonia; stocking density; MAP-K

check for updates

Citation: Mwaura, J.G.; Wekesa, C.; Ogutu, P.A.; Okoth, P. Whole Transcriptome Analysis of Differentially Expressed Genes in Cultured Nile Tilapia (*O. niloticus*) Subjected to Chronic Stress Reveals Signaling Pathways Associated with Depressed Growth. *Genes* **2023**, *14*, 795. https://doi.org/10.3390/genes14040795

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1. Introduction

Fish is a crucial dietary component that accounts for 17% of animal protein and 7% of all the proteins consumed globally [1]. Modern advances in fish genetics, nutrient-rich diets, culture methods, and management techniques have made aquaculture one of the world's fastest-growing food production industries [2]. Production of tilapia has quadrupled in the last decade, making it the second most farmed fish worldwide [3]. The goal of aquaculture is to enhance production and productivity, which therefore hints at a possible form of raising process intervention [4]. Some of the factors that are highly manipulated include stocking densities, feeds, feeding and genetics [2]. According to projections, the global fish supply will rise from 179 million tons in 2018 to 204 million tons in 2030. Aquaculture production is anticipated to reach 109 million tons this year with the fastest growth expected for tilapia and shrimps [5,6].

Fish get stressed when their environment's physical and chemical characteristics become unfavorable, e.g., extremes of temperature, pH, salinity, oxygen, ammonia, turbidity and water hardness. Improper fish handling techniques, harassment from other fish and predators, overcrowding, and inadequate nutrition are significant stressors for fish in ponds [7,8]. Exposure of fish to such conditions has been shown to trigger acute or chronic

APPENDIX 6: MANUSCRIPT 2

Cortisol Signatures in Cultured Nile Tilapia [*O. niloticus]* Regulates Mitogen Activated Protein Kinase (MAP K) Signalling Pathway to Modulate Chronic Stress

John Gitau Mwaura[1,2], Benjamin Opot[3], Sylvia Milanoi[3], Edwin Mwakio[3], Sandra Khatiebi[1], Clabe Wekesa[1], Philip Ogutu[1], Patrick Okoth[1].

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[3] Kenya Medical Research Institute, P.O. Box: 1578, 40100 Kisumu, Kenya

Abstract

Chronic stress is a major bottleneck to aquaculture production. Scale cortisol has been used in chronic stress determination. Few studies have attempted to investigate how chronic stress affects metabolic pathways in cultured Nile tilapia. The current study investigated the relationship between cortisol signatures of chronic stress in cultured Nile tilapia in relation to regulation of Mitogen Activated Protein Kinase (MAPK) signalling pathway. This pathway is activated via a protein kinase cascade whose efficiency and specificity is regulated by several scaffold proteins. Juvenile Nile tilapia were cultured in the laboratory at different ammonia concentrations and stocking densities for 70 days. Determination of growth performance, stress levels, RNA sequence and differential gene expression were performed. Results of this study indicated a positive correlation between the levels of stressors and the concentrations of blood glucose, plasma cortisol and scale cortisol. There was a significant difference ($p<0.05$) in the mean plasma cortisol levels between ammonia treatments and the control ($p< 0.05$ i.e 4.71 ± 0.52 ng/ml and 6.50 ± 0.83 ng/ml) respectively. The cortisol levels increased concomitantly with the concentration of ammonia. There was also a significant difference in the plasma cortisol levels between the low stocking densities and the high stocking densities. There was a negative correlation between scale cortisol and growth performance in cultured Nile tilapia. Consistent with this, there was a significant down regulation of the MAPK signalling pathway ($padj<0.05$). MAPK pathway is associated with cell proliferation, differentiation, transcription regulation and development. These findings indicate that chronic stress

APPENDIX 7: MANUSCRIPT 3

Mwaura et al.
The Journal of Basic and Applied Zoology (2023) 84:28
https://doi.org/10.1186/s41936-023-00346-6

The Journal of Basic and Applied Zoology

RESEARCH Open Access

Pangenomics of the cichlid species (*Oreochromis niloticus*) reveals genetic admixture ancestry with potential for aquaculture improvement in Kenya

John G. Mwaura[1,2], Clabe Wekesa[1,4], Kiprotich Kelvin[1], Ang'ienda Paul[3], Philip A. Ogutu[1] and Patrick Okoth[1*]

Abstract

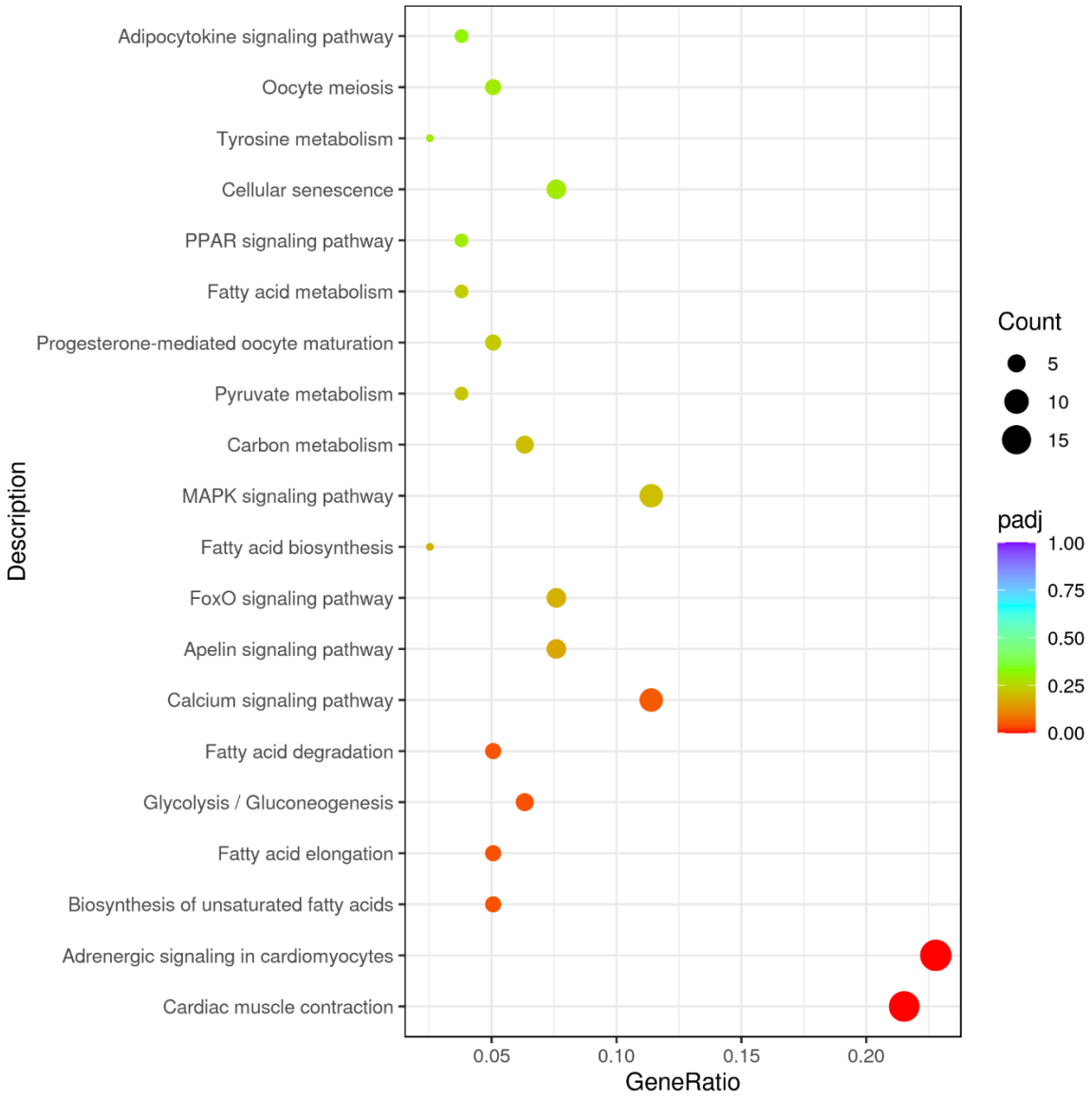
Background Nile tilapia has a variety of phenotypes suitable for aquaculture farming, yet its entire gene pool with potential for breeding climate ready strains in resource-limited settings remains scanty and poorly documented. SNP calling has become increasingly popular in molecular genetic studies due to their abundance and precision in estimating and identifying an organism's genetic makeup. SNPs are versatile in trait-specific breeding, which, in contrast to pedigree breeding, is affordable and speeds up genetic advancement by allowing animals to be used as parents sooner.

Results Clustering analysis revealed a strong correlation between the experimental sample, *Oreochromis niloticus*, *Oreochromis aureus* and *Betta splendens*. Three other species indicated evolutionary independence. Comparative genomics identified similarities between fishes with common genetic and evolutionary ancestry, allowing for better adaptation to local environmental conditions. Some of the selected genes exhibiting substantial effect on immunity include: *Prxs*, *MMR1 like*, *ZMYM4-like partial*; stress-reactive genes including: *PALLD-like* gene, *LPLBAG6-like* and growth-related genes including: *NF1x like*, *PEDF* and *CL like*. *Experimental sample*, *O. niloticus*, *O. aureas and Danio rerio*, can hybridize in their natural environments bringing about genetic admixture ancestry that hybridizes new genes which confer beneficial phenotypes.

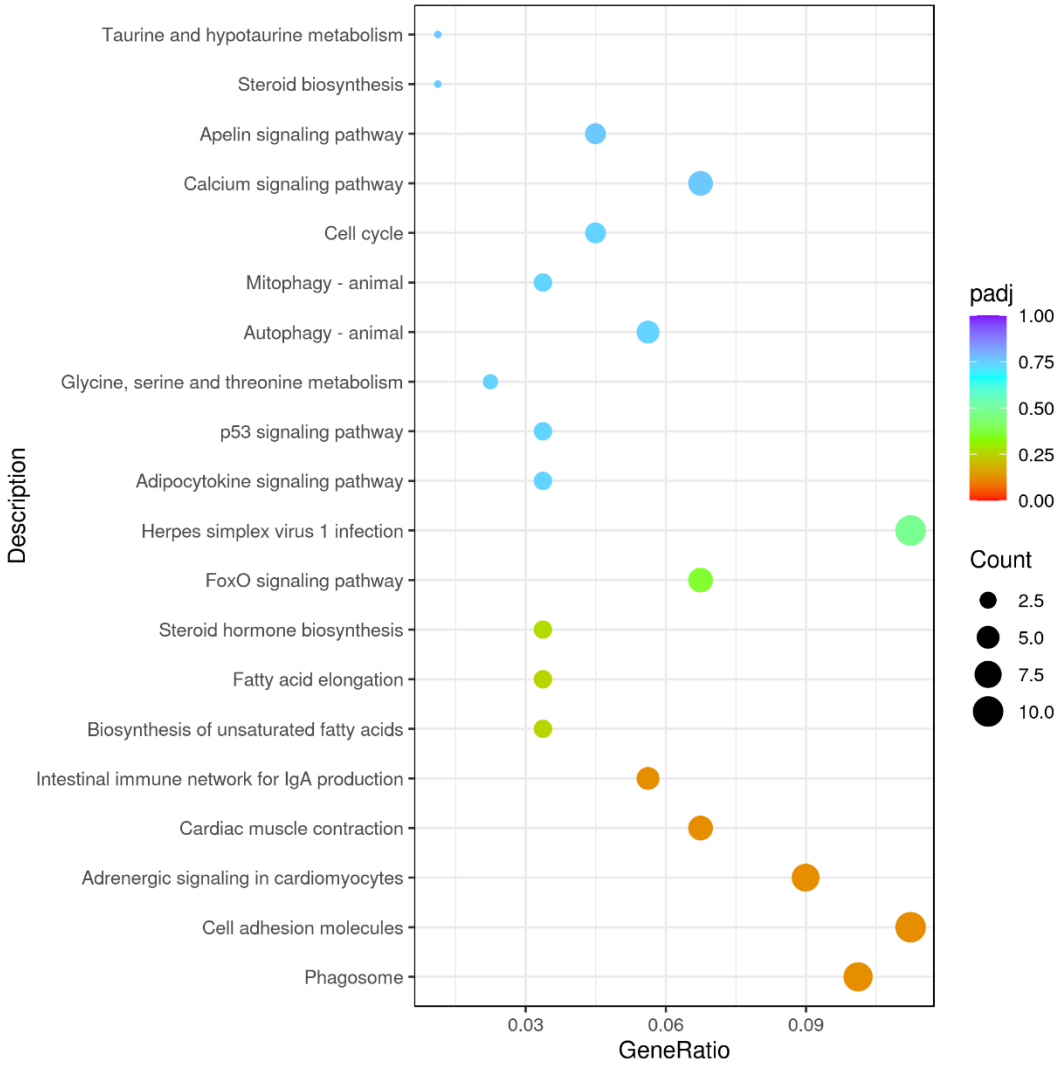
Conclusion Breeding for specific traits could be a useful addition to aquaculture to allow expansion of the ecological niche and genetic base for tilapia. Some of the beneficial genes that can be hybridized include Slc25a24 and *Slc-12member 10*, *tandem duplicate 1*, for salinity tolerance and *Abca1*, *bcl2a and mylk13* for hypoxia tolerance. Breeders should introduce beneficial traits into fish breeds to ensure they are climate ready and able to weather climate shocks. This will allow aquaculture to contribute to food and nutrition security in line with SDG2 and improve the economic status of fish-farming communities in the Global South countries.

Keywords Pangenome, Genetic admixture ancestry, Introgression, Stress, Nile tilapia, Pedigree breeding

APPENDIX 8: AMMONIA TREATMENT: UP AND DOWN REGULATED PATHWAYS

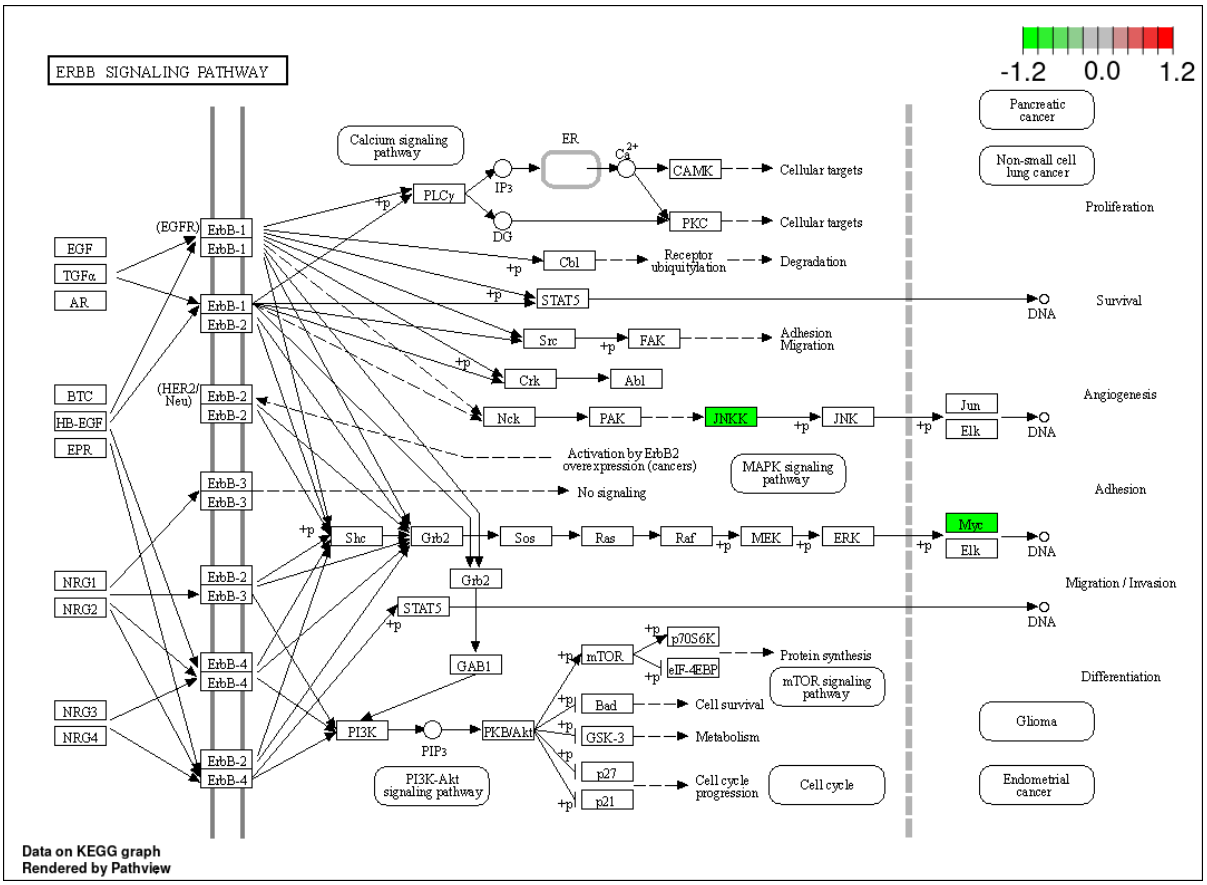


APPENDIX 9: STOCKING DENSITY TREATMENT: UP AND DOWN REGULATED PATHWAYS

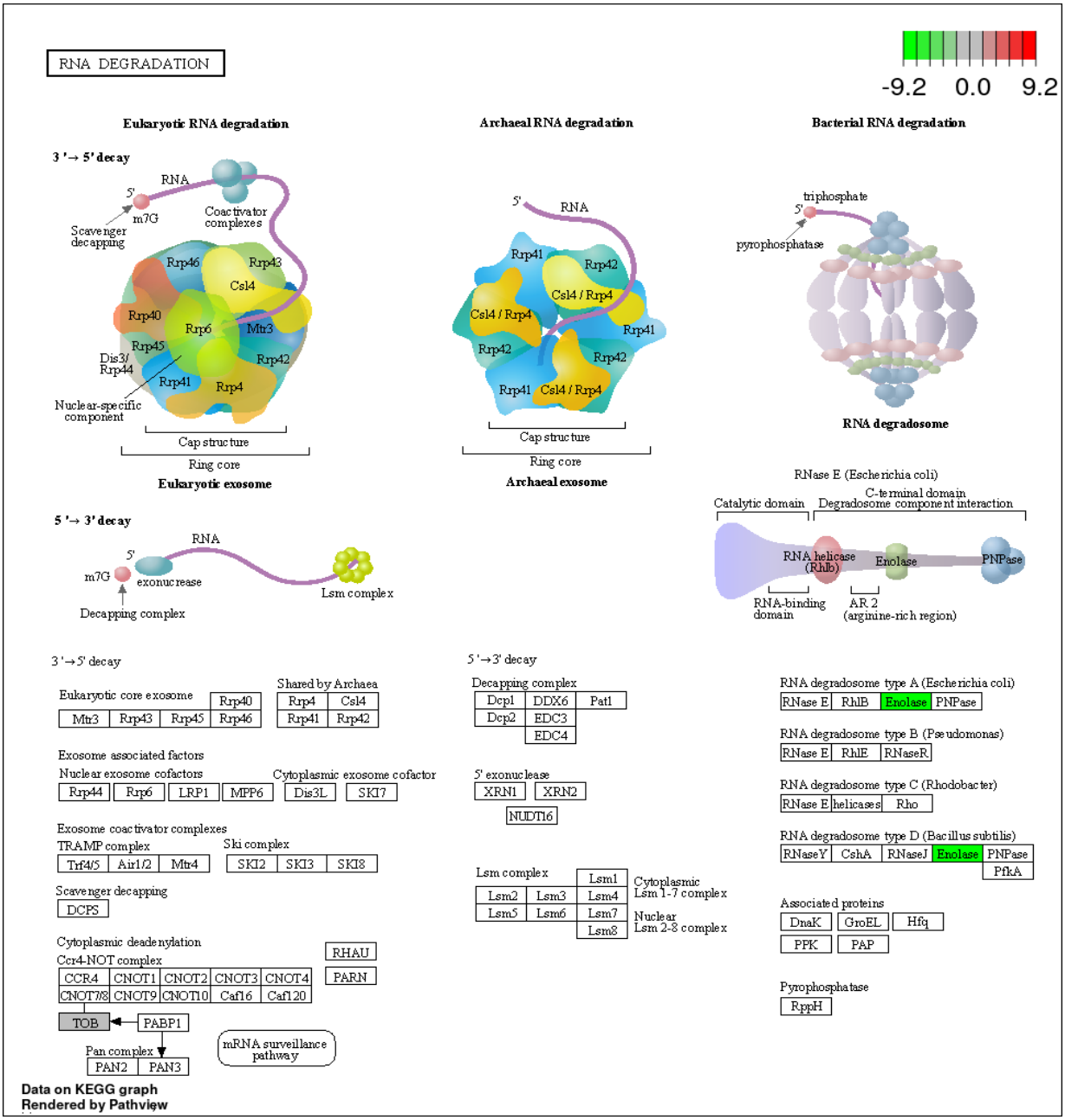


APPENDIX 10: AMMONIA DOWNREGULATED PATHWAYS

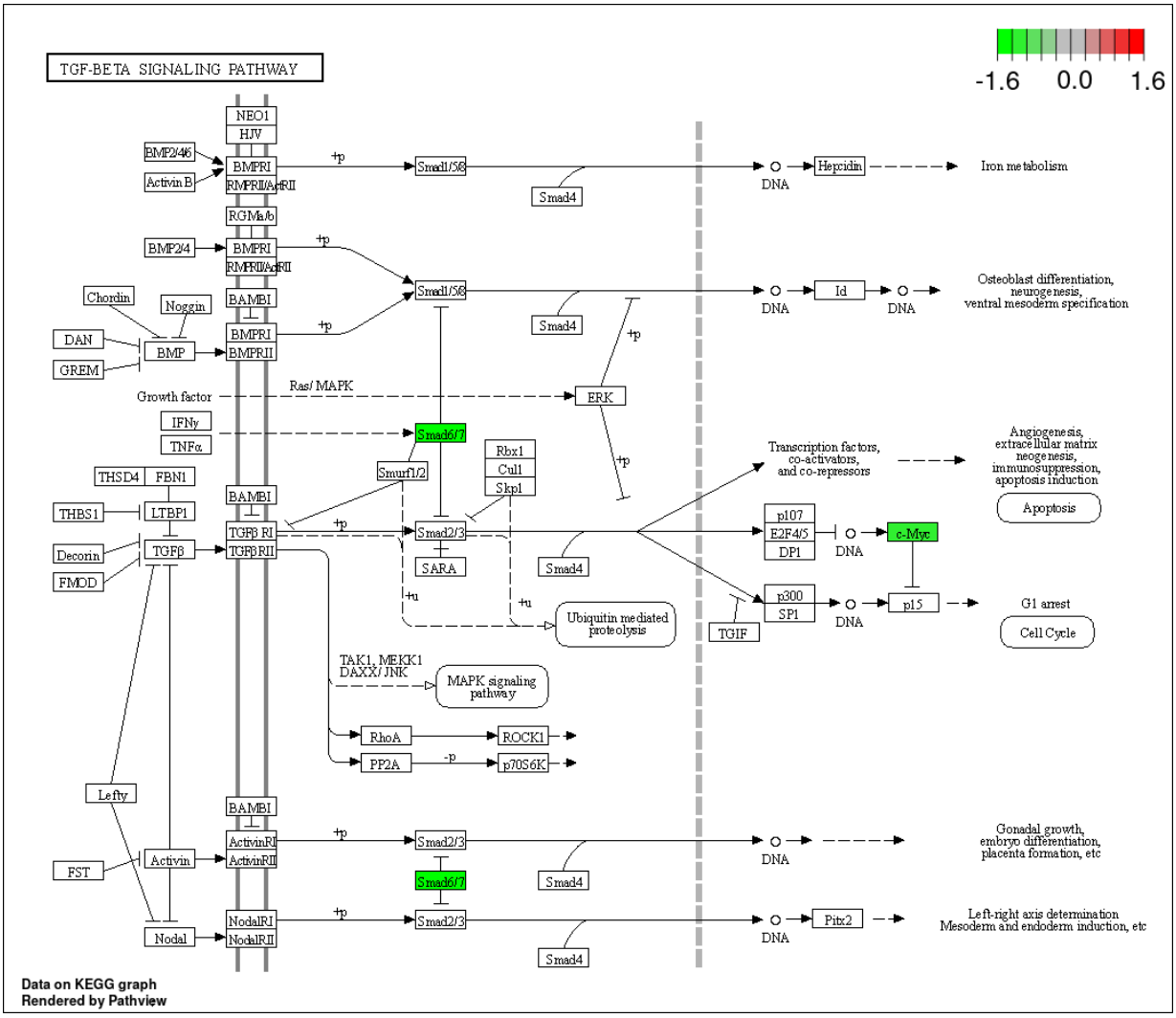
a) ERB signaling pathway



b) RNA Degradation

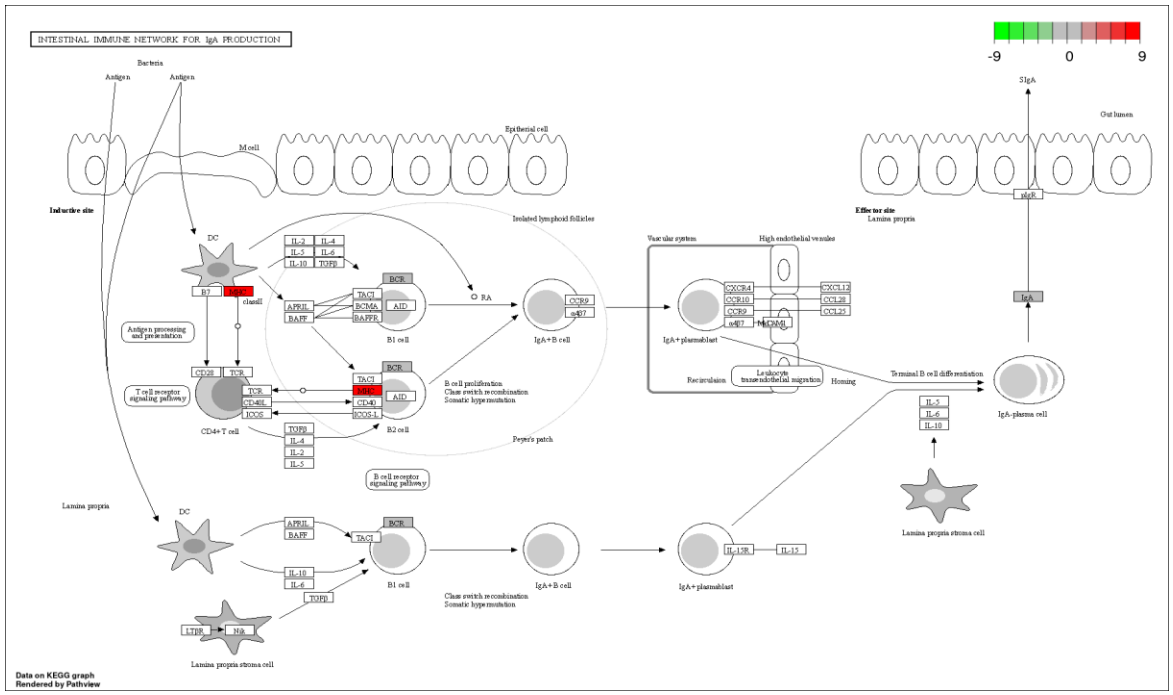


c) TGF-Beta signaling pathway

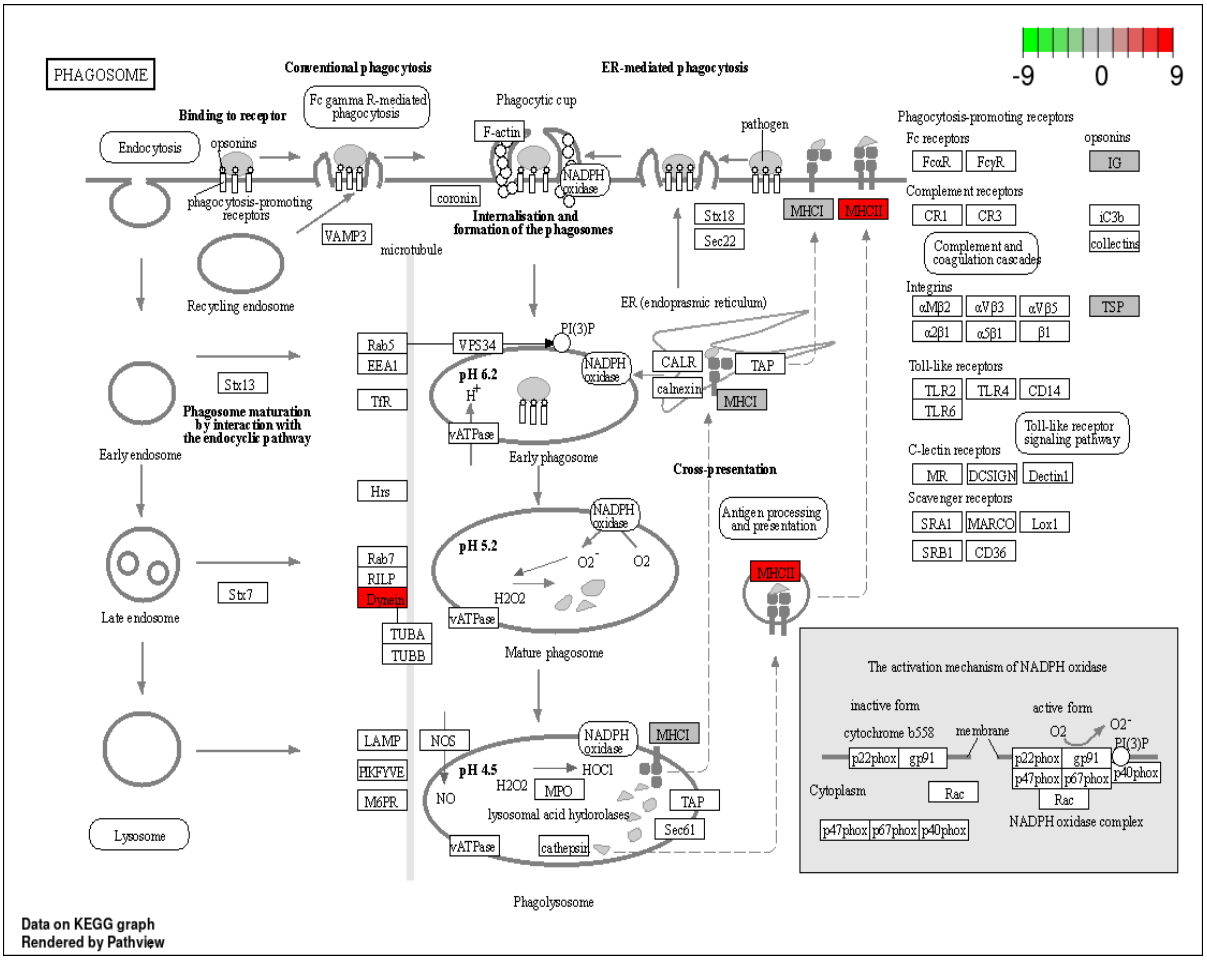


APPENDIX 11: STOCKING DENSITY UPREGULATED PATHWAYS:

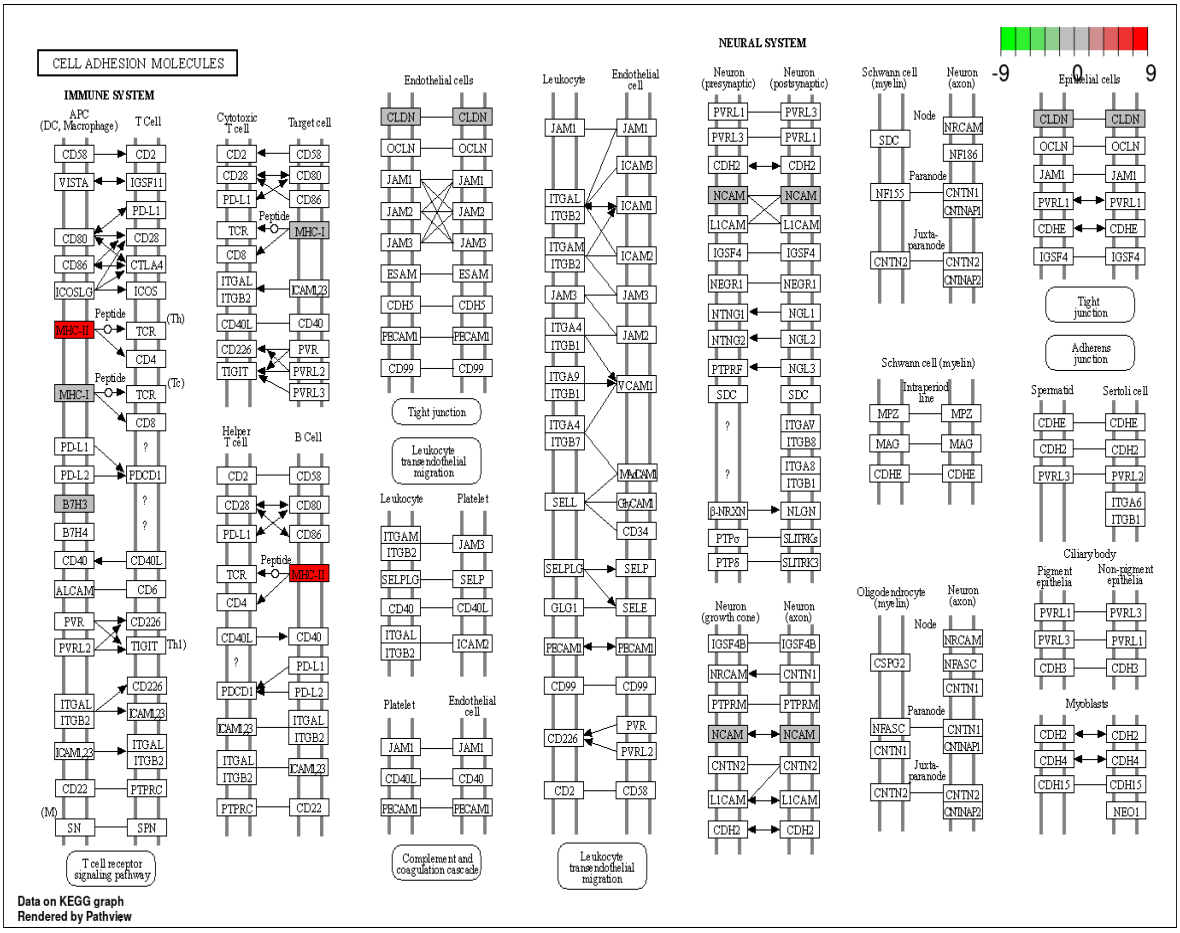
a) Intestinal immune network for Iga production



b) Phagosome


PHAGOSOME
Conventional phagocytosis
ER-mediated phagocytosis
-9
0
9
Binding to receptor
Fc gamma R-mediated phagocytosis
Phagocytic cup
F-actin
pathogen
Endocytosis
opsonins
phagocytosis-promoting receptors
VAMP3
coronin
NADPH oxidase
Internalisation and formation of the phagosomes
microtubule
Stx18
Sec22
MHCI
MHCII
Phagocytosis-promoting receptors
Fc receptors
FcαR
FcγR
opsonins
IG
Complement receptors
CR1
CR3
iC3b
collectins
Complement and coagulation cascades
Integrins
αMβ2
αVβ3
αVβ5
TSP
α2β1
α5β1
β1
Toll-like receptors
TLR2
TLR4
CD14
TLR6
Toll-like receptor signaling pathway
C-lectin receptors
MR
DCSIGN
Dectin1
Scavenger receptors
SRA1
MARCO
Lox1
SRB1
CD36
Recycling endosome
ER (endoprasmic reticulum)
Rab5
EEA1
VPS34
PI(3)P
NADPH oxidase
CALR
TAP
calnexin
MHCI
pH 6.2
H+
TfR
vATPase
Early phagosome
Stx13
Phagosome maturation by interaction with the endocytic pathway
Early endosome
Cross-presentation
Antigen processing and presentation
Hrs
NADPH oxidase
pH 5.2
O2-
O2
H2O2
Rab7
RILP
Dynein
vATPase
MHCII
Stx7
Late endosome
TUBA
TUBB
Mature phagosome
The activation mechanism of NADPH oxidase
inactive form
active form
cytochrome b558
membrane
O2
O2-
PI(3)P
p22phox
gp91
p47phox
p67phox
p40phox
Cytoplasm
Rac
NADPH oxidase complex
LAMP
NOS
NADPH oxidase
MHCI
HKFYVE
pH 4.5
HOCl
H2O2
MPO
NO
TAP
M6PR
lysosomal acid hydrolases
Sec61
vATPase
cathepsin
Lysosome
Phagolysosome
Data on KEGG graph
Rendered by Pathview

c) Cell adhesion molecules



APPENDIX 12: WHOLE GENOME SEQUENCE

Oreochromis niloticus breed Nile tilapia isolate F1, whole genome shotgun sequencing project

GenBank: JANFCW000000000.1

This entry is the master record for a whole genome shotgun sequencing project and contains no sequence data.

Go to:

```
LOCUS       JANFCW010000000      1017644 rc    DNA     linear   VRT 03-OCT-2022
DEFINITION  Oreochromis niloticus breed Nile tilapia isolate F1, whole genome
            shotgun sequencing project.
ACCESSION   JANFCW000000000
VERSION     JANFCW000000000.1
DBLINK      BioProject: PRJNA848236
            BioSample: SAMN28990247
KEYWORDS    WGS.
SOURCE      Oreochromis niloticus (Nile tilapia)
  ORGANISM  Oreochromis niloticus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Neoteleostei;
            Acanthomorphata; Ovalentaria; Cichlomorphae; Cichliformes;
            Cichlidae; African cichlids; Pseudocrenilabrinae; Oreochromini;
            Oreochromis.
REFERENCE   1  (bases 1 to 1017644)
  AUTHORS   Mwaura,J.G., Wekesa,C.S., Ogutu,P. and Okoth,P.
  TITLE     Nile Tilapia draft genome
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1017644)
  AUTHORS   Mwaura,J.G., Wekesa,C.S., Ogutu,P. and Okoth,P.
  TITLE     Direct Submission
  JOURNAL   Submitted (25-JUN-2022) Biological Science department, Masinde
            Muliro University of Science and Technology, Kisumu-Webuye Road,
            Kakamega, Western 50100, Germany
COMMENT     The Oreochromis niloticus whole genome shotgun (WGS) project has
            the project accession JANFCW000000000.  This version of the project
            (01) has the accession number JANFCW010000000, and consists of
            sequences JANFCW010000001-JANFCW011017644.

            ##Genome-Assembly-Data-START##
            Assembly Method        :: SOAPdenovo v. 2.40
            Genome Representation  :: Full
            Expected Final Version :: No
            Genome Coverage        :: 37.0x
            Sequencing Technology  :: Illumina MiSeq
            ##Genome-Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..1017644
                     /organism="Oreochromis niloticus"
                     /mol_type="genomic DNA"
                     /isolate="F1"
                     /db_xref="taxon:8128"
                     /sex="female"
                     /tissue_type="myotomes"
                     /note="breed: Nile tilapia"
WGS         JANFCW010000001-JANFCW011017644
```