

**DIETARY EFFECTS OF *ARTEMISIA AFRA* ON STRESS RESPONSE IN
OREOCHROMIS MOSSAMBICUS AND *CLARIAS GARIEPINUS***

by

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RESEARCH DISSERTATION

Submitted for the fulfilment of the requirements for the degree of

MASTER SCIENCE

in

AQUACULTURE

in the

FACULTY OF SCIENCE AND AGRICULTURE

(School of Agriculture and Environmental Sciences)

at the

UNIVERSITY OF LIMPOPO

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2025

DEDICATION

In loving memory of my sister (Thelma Moloto), uncle (Mackson Madikana Mothompeni), and aunt (Pholo Annah Masipa). May your souls continue to rest in eternal peace.

Overall, this masterpiece is dedicated to my lovely grandmother, Mosusumedi Moloto. Thank you for all your teachings and guidance.

1 Thessalonians 5:18

DECLARATION

I declare that **DIETARY EFFECTS OF *ARTEMISIA AFRA* ON STRESS RESPONSE IN *OREOCHROMIS MOSSAMBICUS* AND *CLARIAS GARIEPINUS*** dissertation hereby submitted to the University of Limpopo, for the degree of **MASTER OF SCIENCE IN AQUACULTURE** has not been previously submitted by me for credit toward a degree at this university or any other; that it is entirely my own work, both conceptually and technically; and that all sources have been properly cited.



Maripa Herold Moloto (Mr.)

04/06/2025

Date

PLAGIARISM DECLARATION

The dissertation has been submitted in through the Turnitin module, and I certify that my supervisors have viewed my report and that any concerns identified have been discussed with them.



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04/06/2025

Date

ACKNOWLEDGEMENTS

My deepest appreciation to God, the creator of all that exists, for providing me the gift of life and for keeping me alive and guarding me throughout the entire study period. I sincerely appreciate Him for the strength He provided me to complete this research. Jehovah God of Mount Zion always has a ton of creative ideas. You are such a magnificent God! I owe my gratitude to all these people.

- Prof. N.A.G. Moyo, my main supervisor, for his insightful criticism, the ability to review and modify my work, and his fatherly and analytical supervision. I came to the Aquaculture Research Unit as a small boy, knowing absolutely nothing about Aquaculture, but under your wing, I became a big man and proud to say that I am your product. The knowledge, encouragement, and enthusiasm you have stimulated me to have a passion and love for fish biology, health, and nutrition as a whole in any aspect of academic life. Thank you, Prof.
- My co-supervisor, Dr. E.M. Mbokane, for his unwavering commitment to co-supervising this research, his fair and firm remarks throughout the study, and his role as a good listener and advisor when needed is appreciated “*Mei Doc*”.
- Prof. J.P. Maree for encouraging me to continue with my studies and his willingness to see me following my dreams and seeing the potential within me since my undergraduate studies.
- Prof. M.M. Mphahlele-Makgwane from the Water and Sanitation Department is recognized for her unquestioning belief in my abilities even when situations were overwhelming at times, her love and constructive sessions where I had to grow and be a novice scientist I have always wished to be.
- The technician for the Aquaculture Research Unit, Mr. G. Geldenhuys, and his team (Mma Eva, Sesi Clara, Hector, and KG) are honoured for their technical assistance during the fish stressing trials.
- I am very much grateful to Prince Sello Molokomme who shared the same pain and laughter with me as we had the similar workloads working with freshwater species in our Master Science studies, although in different departments. Your insightful words and guidance throughout this journey are appreciated my friend.
- My wonderful ARU colleagues: Dr. Eva Nephale-Mabidilala, Dr. Cathrine Hlongwane, Selaelo Mphalo, Fiona Mashele, Mahuma Ribane, Central

Makhuiting and Andries Rammala, I didn't only find in you guys' analytical advice only, but big brothers and sisters who always knew how to pick me up when the hope was finished, Thank you.

- To my parents, Simon Mahlodi "Bra Shloks" Moloto and Kate Masalla Moloto, I owe you everything and I appreciate you for instilling me with good morals, intellectual ability, accountability, and good health. Without you, I would not have been where I am today. "*Ke lebogile Batswadi Baka!*"
- To my little sister (Selae Moloto) and nephew (Nhlamulo Moloto), and also my cousins Lillian, Conny, Macheba, Makole, Pholoso, Maria, Khomotjo, and Koketso for always being part of my life even in difficult times, you guys made me to push through limits.
- I am grateful for Mrs Makoma Shirly Motsai and Mrs Selina Serole Lephale for being one of the best aunts the world can ever ask for, I am taking this home with you my loved ones.
- My awesome uncles Stephen Moloto, George Rasweswe, Tshepo Rasweswe, and Andrew Masipa continue being the best in everything you do guys. You are very much appreciated.
- My extraordinary sister from another mother, Sanah Sekedi Malemela for always believing in me and ensuring that I am well taken care of when I am not feeling well.
- My friends from who made my stay at UL welcoming and yet wonderful, Thabang, Koketso, Thabo, Trudy, Karabo, Dorcus, and Sello.
- Thato Kelvin Ledwaba, known as "Kelvin Momo", for creating calming yet profound music that helped me stay sane, particularly during the research experiments I carried out more especially on weekends alone for this dissertation.
- I am very much thankful for the funds received from the Department of Forestry, Fisheries and Environment (DFFE), ROC Water Technologies and French South African Agriculture Institute (F'SAGRI).

ABSTRACT

The aim of this study was to investigate the anti-stress properties of the *A. afra* essential oil in *Oreochromis mossambicus* and *Clarias gariepinus* subjected to different stressors, namely handling, feed deprivation and stocking density. Ten percent (10%) *Artemisia afra* was reported to boost immunity in *O. mossambicus* and *C. gariepinus*. *Artemisia afra* contains active ingredients such as polyphenols, phenols, tannins, saponins, flavonoids, carotenoids, alkaloids, and Vitamin C, compounds with various functions such as antioxidant properties. Two diets D1 (0%) and D2 (10%) *A. afra* were formulated and randomly fed to triplicate groups of *Oreochromis mossambicus* (30.65 ± 4.61 g) and *Clarias gariepinus* (38.36 ± 4.89 g) stocked at 15 fish per treatment in a 1000 L tank, filled to a 900 L mark. Fish were fed twice daily (09:00hr and 15:00hr) until apparent satiation.

The effect of handling stress on the opercular beat rate, growth performance, and innate immunity in *O. mossambicus* and *C. gariepinus* fed with *A. afra*-based diets was investigated over a period of 45 days. Different fish species respond differently to handling times, therefore, handling times in *O. mossambicus* were control (0), 10, 15, and 20 minutes. In *C. gariepinus*, the handling times were control (0), 15, 30, and 45 minutes. All fish were scooped out of the tank and were handled by gripping the whole body twice daily (09:00hr and 15:00hr) for 3 days a week. Control denotes that there was no fish handling. The results indicated that an increase in opercular beat rate was much higher ($P < 0.05$, ANCOVA) in fish fed with the 0% *A. afra*-based diet than in fish fed with a 10% *A. afra*-based diet. This may indicate that fish fed with the 0% *A. afra*-based diet were more reactive to handling, leading to a higher opercular beat rate, which resulted in stress. Specific growth rate and feed intake in both fish species decreased significantly ($P < 0.05$, ANCOVA) in fish fed with the 0% *A. afra*-based diet than in fish fed with the 10% *A. afra*-based diet. The highest SGR and lowest FCR were observed in tilapia handled for 20 minutes and catfish handled for 45 minutes. Mortality rates also showed that 10% *A. afra*-based diet possesses compounds that assist with improving growth as there were no mortalities in fish fed with the 10% *A. afra*-based diet. This shows the positive effect of supplementing 10% *A. afra*-based diet in the diets of both fish species. The best plasma cortisol and blood performance were observed in fish fed with the 10% *A. afra*-based diet than in fish fed with the 0%

A. afra-based diet; and it was denoted in *O. mossambicus* handled for 20 minutes and *C. gariepinus* handled for 45 minutes. Lysozyme activity was not influenced ($P > 0.05$, ANCOVA) by handling times in both fish species fed with the 10% *A. afra*-based diet, whereas lysozyme activity in fish fed with the 0% *A. afra*-based diet significantly declined with increasing handling times ($P < 0.05$, ANCOVA). This indicates that there was high resistance against handling, thus strengthening the body's ability to fight against stressors and diseases in fish fed with the 10% *A. afra*-based diet. These results indicated that the opercular beat rate, growth performance, and innate of *O. mossambicus* and *C. gariepinus* fed with the 10% *A. afra* were not affected by handling stress. Therefore, the null hypothesis is accepted.

The second experiment was conducted to evaluate the effect of feed deprivation periods on the opercular beat rate, growth performance, and innate immunity in *O. mossambicus* and *C. gariepinus* fed with 0% and 10% *A. afra* inclusion levels over a period of 45 days. Different fish species respond differently to feed deprivation periods, therefore, feed deprivation periods in *O. mossambicus* were control (0), 3, 6, and 9 days whilst feed deprivation periods in *C. gariepinus* were control (0), 6, 12 and 18 days. Control denotes that there was no feed deprivation. The opercular beat rate in feed deprivation followed the same trend as handling stress ($P < 0.05$, ANCOVA). Fish fed with the 10% *A. afra*-based diet recorded the best growth performance (SGR, FCR and feed intake) than fish fed with the 0% *A. afra*-based diet. The best growth performance was observed in *O. mossambicus* and *C. gariepinus* deprived of feed for 6 days and 12 days, respectively. This may indicate that 10% *A. afra*-based diet helped in maintaining gut health during the 6 days and 12 days of feed deprivation period in both species, which allowed better digestion and higher feed intake when the food was reintroduced. The results on mortality rates were similar to handling stress. Plasma cortisol was not influenced ($P > 0.05$, ANCOVA) by feed deprivation periods in both fish species fed with the 10% *A. afra*-based diet. This may explain why plasma cortisol levels did not rise when fish were under feed deprivation stress. The best glucose levels and blood performance were observed in *O. mossambicus* deprived of feed for 6 days and *C. gariepinus* deprived of feed for 12 days fed with the 10% *A. afra*-based diet. Lysozyme activity followed the same trend as handling stress. The results of this experiment demonstrated that the opercular beat rate, growth performance, and innate

of *O. mossambicus* and *C. gariepinus* fed with the 10% *A. afra* were not affected by feed deprivation periods. Additionally, it shows that the 10% *A. afra*-based diet improved better feed intake, thus affecting growth. Therefore, the null hypothesis is accepted.

The last experiment evaluated the effect of stocking density on the opercular beat rate, growth performance, and innate immunity in *O. mossambicus* and *C. gariepinus* fed with 0% and 10% *A. afra*-based diets over a duration of 21 days. The stocking density experiment was limited to 21 days. This shorter duration was sufficient to observe the acute effects of elevated stocking density, such as immediate stress responses, increased competition for resources, and rapid changes in growth performance or opercular beat rate. The fish were stocked in 500 L fibreglass tanks in a completely randomized design. The fish stocking densities for both fish species were as follows; low stocking density (LSD): 1.44 kg/m³ (45 fish/treatment), medium stocking density (MSD): 2.63 kg/m³ (75 fish/treatment), and high stocking density (HSD): 3.68 kg/m³ (105 fish/treatment). The experimental system used for both fish species was the same. In *O. mossambicus* fed with the 10% *A. afra*-based diet, the best ($P < 0.05$, ANCOVA) SGR, feed intake, FCR, and lowest opercular beat rate were recorded at high stocking density (3.68 kg/m³). This suggest that 10% *A. afra*-based diet maximized feed intake and SGR while minimizing the effects of stress and fish were able to convert feed into body mass efficiently, leading to optimal growth at high stocking density. The highest SGR, feed intake, best FCR and lowest opercular beat rate in *C. gariepinus* fed with the 10% *A. afra*-based diet were recorded at medium stocking density (2.63 kg/m³) ($P < 0.05$, ANCOVA). At medium stocking densities, there was no competition for food and space and as a result, fish stress did not occur. The fish may have been able to channel their energy to feeding instead of fighting stress. Mortality rates were similar to that observed in handling stress and feed deprivation experiments In *O. mossambicus*, fish fed with the 10% *A. afra*-based diet recorded the best ($P < 0.05$, ANCOVA) plasma cortisol, glucose levels compared and blood performance than fish fed with the 0% *A. afra*-based diet. The lowest plasma cortisol, glucose and blood performance in *C. gariepinus* was recorded in fish fed with the 10% *A. afra*-based diet than in fish fed with the 0% *A. afra*-based diet at medium stocking density (2.63 kg/m³). This may indicate that the 10% *A. afra*-based diet

inclusion may have helped in stabilizing osmotic balance and reducing physiological stress, leading to lower cortisol and glucose levels. These results indicated that the opercular beat rate, growth performance, and innate of *O. mossambicus* and *C. gariepinus* fed with the 10% *A. afra* were not affected by stocking density. Hence, the null hypothesis is accepted.

The study showed that *Artemisia afra* essential oil have the potential to reduce stress when supplemented in the diets of two commonly warm freshwater species when subjected to handling stress, feed deprivation and stocking density. Reducing the stocking density is recommended to maximize the growth and innate immunity of catfish at high densities. While high stocking densities are often necessary in catfish culture to reduce cannibalism, particularly during early developmental stages; excessively high densities can lead to negative impacts on growth performance, water quality, and immune function. Therefore, stocking density must be carefully optimized to balance the reduction of cannibalism with the maintenance of physiological health and growth. Moderately high densities may provide a compromise by minimizing cannibalism without excessively compromising innate immunity and growth. At farm levels, fish can be handled and deprived of feed without adversely affecting the growth and health of fish when supplemented with *A. afra* essential oil at a concentration of 10%. This may increase warm freshwater aquaculture production and improve the livelihood of local fish farmers.

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LIST OF ABBREVIATIONS

<i>A. afra</i>	<i>Artemisia afra</i>
ANCOVA	Analysis of Variance
<i>A. vera</i>	<i>Aloe vera</i>
BP	Blood performance
<i>C. carpio</i>	<i>Cyprinus carpio</i>
<i>C. gariepinus</i>	<i>Clarias gariepinus</i>
FCR	Food Conversion ratio
<i>H. longifilis</i>	<i>Heterobranchus longifilis</i>
HGB	Haemoglobin
HCT	Haematocrit
HSD	High stocking density
<i>I. Punctatus</i>	<i>Ictalurus punctatus</i>
LSD	Low stocking density
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MSD	Medium stocking density
<i>O. mykiss</i>	<i>Oncorhynchus mykiss</i>
OBR	Opercular beat rate
OBR/min	Opercular beat rate per minute
<i>O. mossambicus</i>	<i>Oreochromis mossambicus</i>
<i>O. niloticus</i>	<i>Oreochromis niloticus</i>

RAS	Recirculating Aquaculture System
RBC	Red blood cells
SGR	Specific growth rate
SPSS	Statistical Package for the Social Sciences
WBC	White blood cell

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CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1: GENERAL INTRODUCTION

1.1 GENERAL INTRODUCTION

1.1.1 Global status of aquaculture

Aquaculture is the fastest-growing animal food-producing sector in the world, with an overall increase of 4.2% per year (FAO, 2022). Currently, aquaculture accounts for nearly-half (45.6%) of the world's food fish consumption, compared with 33.8% in 2000 (Cai and Leung, 2022). Aquaculture production is projected to reach 109 million tonnes in 2030, an increase of 32% (26 million tonnes) since 2018. China is the largest aquaculture producer, accounting for about 78% of the total production (FAO, 2020). The global dominance of China in aquaculture production is due to its over 2 000-year pro-active government policies promoting aquaculture development in the country (Hishamunda and Subasinghe, 2003). At the continental level, Africa was the fourth largest aquaculture-producing continent in 2018, accounting for 27% of global production (FAO, 2020). Aquaculture production in Africa has increased by 60% since 1998 (FAO, 2022). Egypt is the leading aquaculture producer in Africa, producing about 1 592 000 Metric tonnes in 2020 (FAO, 2022).

1.1.2 Status of aquaculture in South Africa

The freshwater aquaculture industry is one of the sectors that has been expanding over the years. However, in the South African context, marine aquaculture has been an expanding sector since the early 2000s (Moloney *et al.*, 2013). In freshwater aquaculture, tilapia and catfish are the most widely cultured species in South Africa (Moyo and Rapatsa, 2021). Freshwater aquaculture in South Africa has not expanded significantly over the years, especially compared to countries like Egypt, Nigeria, or Asian aquaculture powerhouses. While there have been efforts to develop the sector, the overall growth remains slow and limited. This is due to water scarcity, high costs, limited technical support, and regulatory hurdles. Hence, they hold potential in rural development, job creation, and food security, therefore they have to be addressed through coordinated support. Mozambique tilapia, *Oreochromis mossambicus* (Peters, 1852), is the most widely cultured tilapia species. Fast growth rates, a tolerance to unfavourable environmental conditions, and the capacity to consume a

range of food items are just some of the traits that make *O. mossambicus* easy to culture (El-Sayed, 2006; Nguyen *et al.*, 2009). *Clarias gariepinus* becomes one of the widely cultured fish species in Limpopo Province because of low input cost in maintenance and high output production (El-Sayed, 2006). The other reason is its capacity to endure harsh environmental conditions and its ability to feed on a variety of food items. *Clarias gariepinus* has been introduced to new territories such as the Netherlands, Hungary, South-East Asia, and East Asia (FAO, 2020). *Clarias gariepinus* has been successfully introduced in several countries due to its versatile ability to adapt to new environmental conditions. Catfish of the genus *Clarias* are broadly distributed in Africa and Asia.

1.1.3 Challenges affecting the aquaculture industry in South Africa

Despite the fact that these fish species are widely cultured in South Africa, the farming of *O. mossambicus* and *C. gariepinus* in the small-scale sector is hampered by challenges such as poor animal husbandry practices, genetic stock, expensive quality diets, lack of markets and skills, and inadequate financial planning which often led to stress (Isa, 2019; Mahieu, 2015; Mofokeng, 2024). Stress is one of the major problems in aquaculture because it suppresses the immune system of the fish, making it more susceptible to pathogens and diseases. According to Barton and Iwama (1991), stress is defined as a state of decreased fitness or any external agent that challenges the immunity of any fish or threatens its survival. For instance, freshwater aquaculture has been adversely affected by a variety of stressors, including handling, transportation, hypoxia, feed deprivation, stocking density, and pathogens that have been associated with high mortality rates (Pridgeon and Klesius, 2012; Morash and Alter, 2016). In most cases, these stressors are not avoidable.

In response to a variety of stressors, fish undergo a series of biochemical and physiological changes to compensate for the challenge imposed upon it and thereby cope with stress (Iwama *et al.*, 1998; Conte, 2004; Schulte, 2014). This generalized stress response in fish has been broadly categorized into primary, secondary, and tertiary responses. Primary response is the initial response that represents the perception of an altered state and initiates a neuroendocrine/ endocrine response that forms part of the generalized stress response in fish (Gamperl *et al.*, 1994), whilst

secondary response comprises the various biochemical and physiological effects associated with stress and mediated largely by stress hormones (catecholamines, growth hormones, and insulin). These stress hormones activate several metabolic pathways that result in alterations in blood chemistry and haematology (Vijayan *et al.*, 1994). The tertiary response represents whole animal and population level changes associated with stress (growth performance, reproduction, and behaviour) (Sopinka *et al.*, 2016). The tertiary response is usually analysed by assessing the effect of handling times, feed deprivation periods, and stocking density on growth performance.

It has been shown that there are various direct and indirect quantitative parameters that can be used as indicators of stress in fish (Barton and Iwama, 1991). These parameters include changes in the level of cortisol in the plasma, alterations in immunological levels (lysozyme, cytokines, lectins, phagocytosis, plasma cortisol, and respiratory burst activity), and changes in the haematological parameters (white blood cells, red blood cells, haematocrit, haemoglobin, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelets, and glucose) (Montoya *et al.*, 2017; Tippayadara *et al.*, 2021). The blood concentration of corticosteroid hormones is a major index of stress in fish, and elevated levels of these hormones arise from activation of the hypothalamus-pituitary-internal (HPI) axis (Gabriel *et al.*, 2011). The current study will use haematological parameters and immunological parameters (lysozyme activity and plasma cortisol) to determine stress levels in cultured tilapia and catfish. White blood cells, red blood cells, haematocrit counts, and haemoglobin counts will also be used in this study to calculate blood performance, which can be used as a stress indicator in fish.

In aquaculture, handling and transportation form part of animal welfare to its facilities whereby it is commonly estimated by addressing injury and mortality rates. Physiological responses that are affected by transportation are increased plasma cortisol, glucose, and non-esterified fatty acids (NEFA) levels. Gill histological changes, including chloride cell migration, indicating osmoregulation disturbances. Additionally, elevated oxidative stress markers and heat shock protein expression. Factors such as restlessness and increased surface activity can influence behavioural responses in fish cultured when transported (Refaey and Li, 2018). Akar (2011)

reported that *Oreochromis aureus* transported for 3.5 hours increased plasmatic cortisol levels and decreased haematological parameters.

Hypoxia (low dissolved oxygen levels) is critically important in aquaculture because it directly affects fish health, survival, growth, and overall farm productivity. Santos *et al.* (2016) also showed that *Oreochromis niloticus* exposed to hypoxic stress led to high mortality rates. This shows that fish rely on dissolved oxygen (DO) in water to produce energy through aerobic respiration. Under hypoxic conditions, fish shift to anaerobic metabolism, leading to lactate buildup and acid-base imbalance, which can impair basic physiological functions. In physiological response, activation of hypoxia-inducible factors (HIFs), leading to altered gene expression. Shift to anaerobic metabolism, resulting in lactate accumulation. In addition, Abdel-Tawwab *et al.* (2019) showed that hypoxia can lead to suppressed immune function (plasma cortisol, lysozyme activity, and respiratory burst) and oxidative stress. Hypoxia is often associated with overstocking, overfeeding, and eutrophication (excess nutrients), which require proper management.

Different fish species respond to stress in various ways. Studies showed that handling, feed deprivation, and stocking density have a severe economic impact on freshwater aquaculture species, including Mozambique tilapia and the African sharptooth catfish (Shoko *et al.*, 2014). In the establishment of intensive aquaculture, significant economic losses have often been reported in tilapia and catfish fish farms due to stress caused by handling, feed deprivation, and overstocking (FAO, 2016). Despite *O. mossambicus* and *C. gariepinus* being considered hardy species under intensive culture, they are susceptible to stress, which suppresses their immune response and lead to diseases. Stress can lead to mortalities as it often leads to leading to diseases, leading to significant financial losses. According to Shoko *et al.* (2019), most aquaculture stress outbreaks occur in developing countries where 90% of tilapia and African sharptooth catfish farming takes place, thus affecting the growth of the industry. Aquaculture losses affect small-scale farmers the most. These farmers have little resources, inadequate opportunities to improve management skills, and poor fish husbandry practices (FAO, 2016). Most farmers use culture systems that make it difficult to avoid stress. Most farmers in developing countries use earthen ponds, which

stresses fish, especially in winter. Therefore, there is a need to explore potential pathways to reduce stress.

Several stress-related studies on both warm- and cold-water fish species across the globe have demonstrated that stressors such as handling, stocking density, and feed deprivation significantly affect physiological responses, including growth performance, immune function, and stress markers. In cold-water species such as *Oncorhynchus mykiss* and *Salmo salar*, elevated stocking densities and handling are associated with increased cortisol levels, reduced feed efficiency, and impaired immunity (Pickering and Pottinger, 1989; Ellis *et al.*, 2002; Laursen *et al.*, 2013). Similarly, in warm-water species like *Oreochromis mossambicus* and *Clarias gariepinus*, exposure to chronic stress conditions has been linked to decreased growth performance, reduced immune capacity, and elevated opercular beat rate often used as a non-invasive stress indicator (Akinrotimi *et al.*, 2009; Gabriel *et al.*, 2015).

There are several ways that can be used to minimize stress in the Mozambique tilapia and African sharptooth catfish culture. Supplementing diets with plant-based products has been shown to be effective in reducing stress (Acar *et al.*, 2015). Medicinal plants contain compounds that are known to have various biological functions, such as immune-stimulatory, antibacterial, antifungal, and antiviral activities and antioxidative properties (Bodur *et al.*, 2018; Hoseini *et al.*, 2019). Biological functions such as antioxidant properties make medicinal plants more suitable for use to reduce stress, thus avoiding suppression of the immune system.

Previous studies conducted using medicinal plants have shown that they can reduce stress in aquaculture (Alexander *et al.*, 2010; Van Hai, 2015; Kumar *et al.*, 2020). Gabriel *et al.* (2019) used diets supplemented with *Aloe vera* to feed *C. gariepinus*, which was kept under stressful conditions such as low pH and high temperature. The study found that *A. vera* extracts improved haemato-biochemical indices when compared to fish not fed with *A. vera* extracts and increased some of the haematological indices of *C. gariepinus*. Additionally, Yilmaz *et al.* (2014) conducted an experiment on the effect of dietary allspice, *Pimenta dioica* powder, on *O. mossambicus* kept at low pH. The study found that *P. dioica* improved haemato-

biochemical and immunological parameters at 10 g.kg⁻¹ supplementation after 60 days.

A review by Tadese *et al.* (2022) showed that medicinal plants can enhance growth, immunity and also be used as antistressor. For instance, the use of *Rosmarinus officinale* (rosemary) leaf powder has shown to mitigate the negative effects of crowding stress in *Cyprinus carpio*. Additionally, *R. officinale* improved immunity (serum catalase, alternative complement, nitro blue tetrazolium and lysozyme activities) in *Oreochromis niloticus*. These studies show that rosemary contains compounds that are beneficial for enhancing immunity and mitigating stress. Another plant of interest in freshwater fish farming is ginger (*Zingiber officinale*). Sukumaran *et al.* (2016) improved growth performance in *Labeo rohita*. Five herbal extracts (*Cynodon dactylon*, *Piper longum*, *Phyllanthus niruri*, *Tridax procumbens*, and *Zingiber officinalis*) increased the survival and growth of *Epinephalus tauvina* (Punitha *et al.*, 2008). *Gingko biloba* extracts have been reported to reduce stress and also improve moderate growth in *Oncorhynchus mykiss* induced by diazinon.

Locally, one of the plants that can be used to reduce stress in tilapia and catfish culture is *Artemisia afra*, a member of the Asteraceae family (Figure 1.1). *Artemisia afra* is one of the only sixteen South African medicinal plants that have been partly or fully developed for commercial products (Hübsch, 2014). It is widely distributed in Southern Africa and readily occurs in South Africa, Namibia, Lesotho, eSwatini, and Zimbabwe. Its natural distribution extends northwards into tropical East Africa and North Africa in areas such as Ethiopia (Van Wyk, 2008). *Artemisia afra* is widespread in all provinces of South Africa except the Northern Cape. In the wild, it grows at altitudes between 2000–2440 metres on damp slopes along stream sides and forest margins. *Artemisia afra* grows in thick, bushy, slightly untidy clumps. The stems are thick and woody at the base, becoming thinner and softer towards the top. The upper surface of the leaves is dark green, whereas the undersides and the stems are covered with small white hairs, which give the shrub the characteristic overall grey colour. *Artemisia afra* flowers in late summer, from March to May (Van Wyk, 2008).



Figure 1. 1: *Artemisia afra* (taken at the University of Limpopo, Botanical Garden, Limpopo Province, South Africa).

Artemisia afra contains several bioactive compounds, such as tannins, saponins, phenolics, polyphenolics, essential oils, and alkaloids. In addition to these compounds, the essential oil of *A. afra* is known to contain the compounds α -pinene, γ -terpinene, camphene, p -cymene, 1.8-cineole, α -thujone, β -thujone, camphor, borneol, Artemisia ketone, and sesquiterpene- 1-3 (Alhassan, 2017). These compounds possess various biological properties. This suggests its use in fish can potentially minimize stress. However, the use of this plant in reducing stress in fish has not been investigated before. Studies conducted by Mbokane and Moyo (2018a, 2018b, 2020a) showed that *A. afra*-based diets at a concentration of ten percent (10%) can possibly be used to enhance fish health.

1.2 PROBLEM STATEMENT

Fish farming involves the rearing of large numbers of fish at densities that normally exceed those commonly found in nature. This often creates a stressful environment for cultured fish, which can lead to poor growth rates and increased susceptibility to infectious diseases (Mbokane and Moyo, 2020a). *Oreochromis mossambicus* and

Clarias gariepinus suffer from several stressors such as overcrowding, transportation, handling, low dissolved oxygen, and higher ammonia levels all of which can affect fish performance (both growth and reproduction) and lead to increased susceptibility to diseases. This can affect the viability of enterprises as it may lead to financial losses. Conte (2004), Assefa and Abunna (2018) reported that fish farmers generally spend over 60% of operational costs on feed and the management of some of the conditions causing stress in fish. Farmers in the small-scale sector are usually affected the most because they are often under-resourced and not well-trained in basic animal husbandry practices. Although stress in cultured fish can be easily managed by following basic animal husbandry, in many culture systems, this is not possible. Therefore, farmers must find sustainable ways to manage stress in fish culture to boost production.

1.3 RESEARCH JUSTIFICATION

The justification of the study is that freshwater fish farmers experience significant economic losses due to disease outbreaks that have affected tilapia and catfish culture. Studies indicate that the freshwater aquaculture sector, which includes tilapia and African sharptooth catfish farming, has been severely impacted economically by bacterial, fungal, and viral infections as a result of stress, and this has led to a statistical decline in the production over the past decade (Richard, 2011; Adeleke *et al.*, 2020; Moyo and Rapatsa, 2021). The most common diseases in tilapia and catfish culture are caused by *Aeromonas hydrophila* (Rodríguez *et al.*, 2008; Pridgeon and Klesius, 2011), *Edwardsiella tarda* (Rodríguez *et al.*, 2008; Lee and Wendy, 2017) and *Streptococcus iniae* (Chen *et al.*, 2012), *Saprolegnia* (Willoughby and Pickering, 1977), and *Ichthyophthirius multifiliis* (Xu *et al.*, 2012). These cause a huge economic loss in tilapia and catfish establishments, particularly by infecting juveniles, leading to higher mortality rates (Walakira *et al.*, 2014). Studies conducted by Mbokane and Moyo (2018a; 2020a) showed that the inclusion of 10% *A. afra*-based diet improved some innate components and also increased the survival rate in both *O. mossambicus* and *C. gariepinus* challenged with *A. hydrophila*. However, their studies did not investigate the potential of this dosage on reducing stress. To the best of our knowledge, studies the use of *A. afra* on reducing stress with respect to growth parameters and stress

indicators (opercular beat rate, haematological parameters, and immunological parameters) in both *O. mossambicus* and *C. gariepinus* handled, deprived of feed and stocked at different densities could not be found. It is, therefore, imperative to investigate whether the 10% *A. afra*-based diet can improve growth performance and reduce stress levels in cultured tilapia and catfish.

1.4 AIM

To evaluate the anti-stress properties of *Artemisia afra* in *Oreochromis mossambicus* and *Clarias gariepinus* diets exposed to different husbandry practices.

1.5 SPECIFIC OBJECTIVES

The specific objectives were to determine:

1. The effect of *A. afra*-based diets on opercular beat rate, growth performance, and innate immunity in *O. mossambicus* and *C. gariepinus* when exposed to different handling times.
2. The effect of *A. afra*-based diets on opercular beat rate, growth performance, and innate immunity in *O. mossambicus* and *C. gariepinus* when subjected to different feed deprivation periods.
3. The effect of *A. afra*-based diets on opercular beat rate, growth performance, and innate immunity in *O. mossambicus* and *C. gariepinus* reared under different stocking densities.

1.6 NULL HYPOTHESIS

The null hypotheses of the study were:

1. Handling times do not affect the opercular beat rate, growth performance, and innate immunity of *O. mossambicus* and *C. gariepinus* fed with the *A. afra*-based diets.
2. Feed deprivation periods do not affect the opercular beat rate, growth performance, and innate immunity of *O. mossambicus* and *C. gariepinus* fed with the *A. afra*-based diets.

3. Stocking density does not affect the opercular beat rate, growth performance, and innate immunity of *O. mossambicus* and *C. gariepinus* fed with the *A. afra*-based diets.

1.7 STRUCTURE OF THE DISSERTATION

The dietary effects of *Artemisia afra* on the stress response in *Oreochromis mossambicus* and *Clarias gariepinus* were assessed. The dissertation has been divided into seven chapters.

Chapter 1

This chapter introduces the problem and highlights the aim of the study. Specific objectives and the research hypothesis are outlined.

Chapter 2

The literature on the use of medicinal plants in *O. mossambicus* and *C. gariepinus* is explored in this chapter.

Chapter 3

In this chapter, the effect of handling stress on the opercular beat rate, growth performance, haematology, and immunity of *C. gariepinus* and *O. mossambicus* fed with the *A. afra*-based diets is assessed.

Chapter 4

The effect of feed deprivation period on the opercular beat rate, growth performance, haematology, and immunity of *C. gariepinus* and *O. mossambicus* fed with the *A. afra*-based diets is investigated in this chapter.

Chapter 5

This chapter explores how *A. afra*-based diets can improve growth and reduce stress in *O. mossambicus* and *C. gariepinus* at different stocking densities.

Chapter 6

The fundamental findings of the study are outlined in this chapter. It also examines the study's limitations and makes recommendations based on the main results and analysis.

Chapter 7

This chapter lists all references cited in the dissertation.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2: LITERATURE REVIEW

2.1 LITERATURE REVIEW

2.1.1 Mozambique tilapia (*Oreochromis mossambicus*)

Oreochromis mossambicus (Figure 2.1) is one of the species used in this study. It is the second-largest cultured tilapia species globally (Matondang, 2022). Matondang (2022) stated that it is a significant aquaculture commodity, and it has been identified as one of the most desirable fish species since it is highly adaptable to withstand a wide range of environmental conditions, including high stocking densities (FAO, 2022). *Oreochromis mossambicus* can survive at low temperatures and shows relatively fast growth and efficient food conversion (Yin *et al.*, 2024). Although farmers prefer the Nile tilapia (*Oreochromis niloticus*), the extensive permit application process makes it difficult to culture, more especially in the Limpopo Province (FAO, 2020).



Figure 2. 1: Mozambique tilapia (*Oreochromis mossambicus* from Skelton, 2001)

2.1.2 The African Sharptooth catfish (*Clarias gariepinus*)

The second species used in this study is the African sharptooth catfish (Figure 2.2). This species is also one of the most successfully cultured fish species around the globe. Catfish are widely used in aquaculture because they are hardy species with the capability to endure harsh environmental conditions and the ability to feed on a variety of food items. *Clarias gariepinus* has been successfully introduced in several countries due to its versatile ability to adapt to new environmental conditions. Catfish of the genus *Clarias* are broadly distributed in Africa and Asia. Several species of the genus *Clarias* and their hybrids are cultured mainly because of their fast growth rate, disease resistance, and amenability to high-density culture (Prajapati, 2022). In its natural range, *C. gariepinus* is a predatory omnivore that feeds on fish, invertebrates, plant material, plankton, reptiles, and amphibians (Mogorosi, 2019). This makes *C. gariepinus* a good candidate for aquaculture as it can be fed on available feed resources.



Figure 2. 2: African Sharptooth catfish (*Clarias gariepinus* from Skelton, 2001)

2.1.3 Fish husbandry practices in freshwater aquaculture

2.1.3.1 Handling

Handling is an important technique used in aquaculture because it helps fish farmers ensure the quality of the fish in terms of both health and grading. It is regarded as a stress inducer that has a negative effect on aquaculture and has received considerable attention due to its effect on growth performance and innate immunity of fish (Mohapatra *et al.*, 2013). Different fish species have varied tolerance handling times.

The optimum handling time for *O. niloticus* has been reported to be 10 minutes (Camargo-dos-Santos *et al.*, 2021). Ferreira *et al.* (2022) also observed the best growth performance and innate immunity in *O. niloticus* when handled for 10 minutes. In a similar study, Rairat *et al.* (2021) reported that the optimum handling time for *C. gariepinus* was 15 minutes. *Oreochromis mossambicus* and *Clarias gariepinus* handling times differ due to their distinct physiological and behavioural characteristics. African catfish are naturally hardy and adaptable species that can thrive in a wide range of aquatic habitats, including stagnant waters, low-oxygen environments, and fluctuating temperatures. Their hardy nature makes them tolerant to longer handling times than *O. mossambicus*.

Fish size can have an impact on determining the appropriate handling time for fish in fish production systems. According to Camargo-dos-Santos *et al.* (2021), handling *O. niloticus* weighing 23.7 grams for 18 minutes resulted in a decline in growth and increased stress levels. Similarly, Sundell *et al.* (2024) recorded that a handling time of 15 minutes in *O. niloticus* (5.24 g) resulted in a decline in growth performance and increased stress levels. However, Shokr (2011) observed a maximum handling time in *O. niloticus* weighing 40.0 g to be 10 minutes, which did not have a significant effect on growth performance and stress levels. Olopade *et al.* (2022) observed an optimum growth performance and innate immunity in *C. gariepinus* weighing 30.36 grams handled for 20 minutes. However, Basharat *et al.* (2020) showed that a handling time of 30 minutes in *C. gariepinus* weighing (30.14 g) resulted in retarded growth and a decline in innate immunity. The literature shows that smaller fish sizes have lower tolerance than big fish sizes when handled. This may be due to the fact that smaller fish have a higher metabolic rate relative to their body; they require a constant and rapid supply of energy to maintain basic physical activities. Therefore, extended handling times could lead to increased stress and lack of oxygen, making it difficult for them to sustain the necessary physiological processes over an extended period (Portz *et al.*, 2006). To the best of our knowledge, the effect of handling times in *O. mossambicus* and *C. gariepinus* fed with *A. afra* has not been investigated. Therefore, this study will explore how 10% *A. afra*-based diet can be used to reduce stress in *O. mossambicus* and *C. gariepinus* exposed to different handling times. A diet supplemented with 10% *A. afra*-based diet has been recommended by Mbokane and

Moyo (2018a; 2020a) because it improved immunity and enhanced diseases resistance against *A. hydrophila* in the diets of *O. mossambicus* and *C. gariepinus*.

Other studies also investigated the physiological and behavioural responses of several fish species due to handling stress. For instance, Barton (2002) found that species like channel catfish (*Ictalurus punctatus*) and largemouth bass (*Micropterus salmoides*) exhibit significant hormonal and metabolic changes under stress. Similarly, Schreck *et al.* (2001) observed erratic swimming and reduced feeding in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). Other species such as Nile tilapia (*Oreochromis niloticus*) and common carp (*Cyprinus carpio*) have also shown heightened cortisol levels and behavioural changes, including increased aggression or lethargy, when subjected to handling and confinement stress (Barcellos *et al.*, 2001; Conte, 2004).

2.1.3.2 Feed deprivation

Feed deprivation can be an appropriate feeding strategy in aquaculture. Feeding must coincide with the time when the fish are in the most physiologically appropriate state for them to be able to take the food when their appetite is at its best. Many fish farmers deprive their fish of feed due to a lack of feed on their farms. This affects the growth performance of fish and increases mortality rates. An appropriate feed deprivation period reduces feed waste and reduces water quality deterioration while ensuring improved fish growth. Feed deprivation periods vary among fish species. The optimum feed deprivation period for *O. mossambicus* has been reported to be 1 day (Sakyi *et al.*, 2020). El-Araby *et al.* (2020) also observed the best growth performance in *O. niloticus* at a feed deprivation period of 24 hours. However, Abdel-Hameid (2011) stated that the feed deprivation of 10 days in *C. gariepinus* resulted in a decline in growth performance and increased stress levels. Sakyi *et al.* (2020) reported that depriving *O. niloticus* of feed for 7 days resulted in poor growth and increased stress levels. In a similar study, Ebeling and Timmons (2012) reported that depriving *C. gariepinus* of feed for 7 days resulted in the best growth performance and low-stress levels. *Oreochromis mossambicus* are herbivorous fish that can only eat small amounts of food at a time because of their smaller stomachs and limited food storage

capacity (Kasumyan and Levina, 2023; Nyandoto, 2013). Their digestive systems' unique traits, besides the fact that plant materials frequently have lower energy concentrations, indicate that they need to eat more frequently to meet their nutritional needs.

Aquaculture system designs and temperature can also have an impact when determining the appropriate feed deprivation period in fish culture. Earthen ponds are subjected to various weather conditions; when temperatures are low (<14 °C), fish tend to be stressed, especially in winter. Small-scale fish farmers use earthen ponds because they have lower operational costs. However, temperature can affect fish due to seasonal variations. Fish are easily exposed to predators when stocked in earthen ponds. The presence of predators in earthen ponds can significantly affect the physiological and behavioural responses of fish, leading to increased stress, reduced feeding activity, altered habitat use, and impaired growth performance. Fish such as *O. niloticus* may reduce movement and feeding to avoid detection, resulting in lower weight gain and feed efficiency (Brown *et al.*, 2005). Physiologically, predator presence elevates cortisol levels, indicating chronic stress, which can suppress immune function and increase vulnerability to disease (Øverli *et al.*, 2002). In addition, the constant threat of predation can cause fish to cluster in safer pond areas, leading to uneven resource use and reduced water quality in those zones (Ferrari *et al.*, 2010).

Additionally, water quality issues may rise in the earthen pond since it is difficult to control water quality in small-scale fish farming, leading to an increase in diseases and retarded growth. Fotedar (2016) reported that recirculating aquaculture systems (RAS) at a temperature of 27°C improved *O. niloticus* and *C. gariepinus* growth performance and innate immunity deprived of feed for 2 days and 5 days, respectively. This shows that tilapia and catfish are warm freshwater species, and they can tolerate warmer temperatures than cold temperatures. Fish growth performance in earthen ponds versus recirculating aquaculture systems (RAS) depends on several factors, including species, water quality, management practices, and stocking density. Generally, fish such as *O. niloticus* often show better growth in earthen ponds due to more natural conditions, availability of natural food (like plankton), and lower stress levels compared to RAS (El-Sayed, 2006). However, RAS can offer superior control

over environmental parameters, resulting in higher survival rates and consistent production, though growth may be limited by higher stress from stocking density and space restriction (Martins *et al.*, 2010). Therefore, while earthen ponds may support better growth under extensive or semi-intensive conditions, RAS is more efficient for intensive, controlled production.

Other fish farmers use raceways as one of their productions since they constitute a continuous flow of water, which helps to improve oxygen levels and waste removal, promoting healthier fish (Bregnballe, 2022). Alla *et al.* (2015) observed the best growth performance and innate immunity of *H. longifilis* at a feed deprivation period of 5 days. However, it is not recommended for small-scale farmers since it is water quality dependent, which makes it unsuitable for areas with limited water availability (Recanati *et al.*, 2017). It is then for any farming area with limited water and small-scale farmers. Demand feeders can also affect feed deprivation periods by ensuring that fish receive a more consistent and appropriate amount of feed. Demand feeders are automated systems that release feed when fish trigger them, usually by pulling a string or touching a sensor. This gives fish control over their feeding schedule, unlike hand feeding or fixed-time automatic feeders. Attia *et al.* (2012) showed that underfeeding fish can result in growth disparities and increased stress levels. Several studies have been conducted on the effect of feed deprivation periods at fish farms. However, measures to reduce stress have been largely ignored. This study will investigate the effect of feed deprivation periods on growth performance and stress levels in *O. mossambicus* and *C. gariepinus* after feeding with the recommended 10% *A. afra*-based diet.

Several studies have examined the physiological and behavioural responses of various fish species to feed deprivation, highlighting effects such as reduced metabolic rate, altered hormone levels, and changes in activity patterns. For instance, *O. niloticus* and *Cyprinus carpio* show decreased growth rates, reduced plasma glucose, and elevated cortisol during periods of fasting (Ali *et al.*, 2003; Wang *et al.*, 2000). Behaviourally, fish may exhibit increased aggression or hyperactivity in early stages of deprivation, followed by lethargy with prolonged fasting. Similarly, Atlantic cod (*Gadus morhua*) and rainbow trout (*Oncorhynchus mykiss*) have demonstrated

suppressed immune responses and changes in liver metabolism under feed restriction (Navarro and Gutiérrez, 1995; Polakof *et al.*, 2006).

2.1.3.3 Stocking density

The productivity and profitability of any commercial aquaculture unit have a direct relationship with the fish stocking density. In order to achieve better growth and innate immunity, optimum stocking density is a key requirement. Small-scale fish farmers increase stocking densities with the aim of optimizing fish production. However, stocking fish at high densities (4.26 kg/m³ - 16 kg/m³) affects the growth and physiological performance of cultured species even when food needs are fulfilled (Harahap *et al.*, 2023). This is mainly due to the competition for space and food that fish experience at high stocking densities of 4.26 kg/m³ - 16 kg/m³. Stocking fish at low densities (0.14 kg/m³ – 2.21 kg/m³) results in inefficient utilization of space and low yields, while high stocking density leads to chronic stress, which compromises the immune response of fish and makes them susceptible to opportunistic pathogens (Wu *et al.*, 2018). The stress resulting from high stocking density causes physiological changes (Li *et al.* 2021). However, Ni *et al.* (2016) and Qi *et al.* (2016) reported that stocking density can have both negative and positive effects on growth and innate immunity of some species. Although the range of 4.26 kg/m³ to 16 kg/m³ may not be considered high in all aquaculture systems, it can still negatively affect growth and physiological performance in certain contexts, especially in tank-based or recirculating aquaculture systems (RAS) where space and water exchange are limited. Therefore, what is considered a high stocking density must be evaluated in relation to species-specific behavior, system design, and fish size. Therefore, this study aims to reduce stress in both fish species cultured at different densities. Among studies that were conducted on reducing stress on cultured fish included medicinal plants such as *Myrcia sylvatica* and *Lippia alba* that were reported to reduce stress in gilthead sea bream (*Spara aurata*) and silver catfish (*Rhamdia quelen*) (Heldwein *et al.*, 2014; Salbego *et al.*, 2017; Saccol *et al.*, 2018). So far, there is little information on using medicinal plants to reduce stress levels in cultured *O. mossambicus* and *C. gariepinus* at different densities.

Stocking density is a crucial environmental factor in aquaculture that significantly influences the physiological and behavioural responses of cultured fish. Other studies have investigated the physiological and behavioural responses of different fish species to varying stocking densities, revealing stress-related changes such as elevated cortisol levels, impaired growth, reduced feed efficiency, and increased aggression or social hierarchy formation. For example, *O.s niloticus* and *Salmo salar* (Atlantic salmon) exhibit higher cortisol concentrations, decreased specific growth rates, and increased fin damage at high densities (Ellis *et al.*, 2002; Barcellos *et al.*, 2006). In *O. mykiss*, high stocking density leads to suppressed immune function and increased susceptibility to disease (Pickering and Pottinger, 1989). Additionally, behavioural changes such as reduced space use, aggressive interactions, and altered swimming patterns have been documented across multiple species under crowded conditions (Turnbull *et al.*, 2005).

2.1.4 Use of medicinal plants in promoting growth and reducing stress in *O. mossambicus* and *C. gariepinus*

The use of medicinal plants for improved growth and reducing stress in aquaculture is becoming increasingly popular and has attracted a lot of attention globally due to an increase in demand for non-invasive alternatives (Dheeran *et al.*, 2023). Medicinal plants contain various compounds such as polyphenols, carbohydrates, amino acids, flavonoids, alkaloids, tannins, organic acids, volatile oils, polysaccharides, minerals, and vitamins, some of which are necessary for growth and improving immunity or overall wellbeing in fish and other animals (Van Hai, 2015). However, in South Africa, there is limited information on the use of medicinal plants as feed additives in aquafeeds to improve growth and reduce stress in cultured fish. There are numerous literatures on the use of medicinal plants as immunostimulants is that of Harikrishnan (2011), Mbokane and Moyo (2018a, 2020a), and Van Hai (2015).

In aquaculture, plants are a potential alternative to synthetic pharmaceuticals because they produce beneficial physiologically active compounds, including immunological modulation. Some have been reportedly found to have biological properties that can improve the activity of digestive enzymes (Ugwuodo and Nwagu, 2021). Several plants

have been investigated for growth performance (weight gain, specific growth rate, feed intake, and feed conversion ratio) of both *O. mossambicus* and *C. gariepinus*. An investigation conducted by Tiamiyu *et al.* (2021) demonstrated that *Allium sativum* significantly increased *C. gariepinus* weight gain. Gabriel *et al.* (2019) also reported that *Aloe vera* improved growth performance (feed intake), feed utilization (FCR), and protein digestibility in *C. gariepinus*. Yilmaz *et al.* (2013) reported that *Cuminum cyminum* can be used as a supplementary feed for a growth promoter during the first feed of *O. mossambicus*. Hlophe and Moyo (2014a; b) reported that the total replacement of fishmeal with *Cenchrus clandestinus* (Kikuyu grass) leaf meal in the diets of *O. mossambicus* and *C. gariepinus* improved growth performance, feed utilization, and protein digestibility. A study by Gültepe *et al.* (2014) showed that the use of 1.0% of spices such as *Rosmarinus officinalis* (rosemary), *thymus vulgaris* (thyme), and *Trigonella foenum graecum* (fenugreek) leaf powder increased growth performance in *O. mossambicus*.

Since these plants were able to improve growth in these fish species, it shows that these plants have the potential to be included in aquafeeds to improve some of the components of innate immunity in *O. mossambicus* and *C. gariepinus* increase the survival rate against different stressors. There is often a direct correlation between growth and innate immunity in fish when fed medicinal plants; especially those with immunostimulant, antioxidant, and antimicrobial properties (Van Hai, 2005; Tadese 2022). However, this relationship can be positive, neutral, or even negative, depending on factors like dosage, plant species, fish species, and duration of feeding. Alexander *et al.* (2010) reported that immunity was improved in *O. mossambicus* fed with diets containing *Tinospora cordifolia* and also increased survival rates when fish were exposed to *A. hydrophila*. Logambal *et al.* (2000) reported that feeding *O. mossambicus* with diets enriched with *Ocimum sanctum* increased immunity and offered protection against *A. hydrophila*. Gabriel *et al.* (2019) reported that *A. vera* diets enhanced immunity in *C. gariepinus* exposed to different pH levels. According to Yilmaz and Ergün (2014), the administration of *Pimenta dioica* enhanced *O. mossambicus* resistance to disease, as evidenced by a higher survival rate following a challenge with *Streptococcus iniae*. Mbokane and Moyo (2018b) indicated that *Moringa oleifera*-based diets increased immunity in *O. mossambicus* and provided

protection against *A. hydrophila*. Similarly, Mbokane and Moyo (2020b) also showed that the moringa-based diets improved innate immunity and survival in *C. gariepinus* after a challenge with *A. hydrophila*.

Several studies focused on plants such as *Zingiber officinale*, *Thymus vulgaris*, *Citrus limon*, and *Pimenta dioica* to treat diseases and improve fish's immune response against pathogens (Immanuel *et al.*, 2009; Gültepe *et al.*, 2014; Yilmaz *et al.*, 2014; Baba *et al.*, 2016). Therefore, there is a need to use these medicinal plants for re-emerging stress outbreaks caused by poor husbandry practices in South African freshwater aquaculture. As shown in the literature, there is a shortage of information on the most effective ways that can be used to improve growth and reduce stress in *O. mossambicus* and *C. gariepinus* under stressful conditions. This includes handling, feed deprivation, and stocking density, all of which will be the focus of the present study.

2.1.5 An overview of different medicinal plant used on different fish species

The use of medicinal plants in aquaculture has gained widespread interest as a natural and sustainable alternative to synthetic chemicals for promoting fish growth and immunity. Various plant-derived supplements have been shown to enhance appetite, feed utilization, and the immune status of diverse fish species. In *Oreochromis niloticus* (Nile tilapia), *Cyprinus carpio* (common carp), *Oncorhynchus mykiss* (rainbow trout), *Labeo rohita* (rohu), and *Clarias gariepinus* (African catfish), herbs such as garlic (*Allium sativum*), turmeric (*Curcuma longa*), neem (*Azadirachta indica*), and ginger (*Zingiber officinale*) have exhibited immunostimulatory, antioxidant, and antimicrobial effects (Awad and Austin, 2010; Harikrishnan *et al.*, 2011; Citarasu, 2010). These plants contain bioactive compounds like allicin, curcumin, azadirachtin, and gingerol, which help strengthen both innate and adaptive immune responses, increase phagocytic activity, and reduce mortality during bacterial infections such as *Aeromonas hydrophila* and *Edwardsiella tarda* outbreaks. Additionally, enhanced growth performance, including improved specific growth rate and feed conversion ratio, has been reported in species like *Sparus aurata* (gilthead seabream) and *Danio rerio* (zebrafish) following dietary inclusion of herbal extracts (Nya and Austin, 2009;

Reverter *et al.*, 2014). The integration of medicinal plants into aquafeeds thus represents a promising, eco-friendly strategy to enhance fish health, reduce disease outbreaks, and boost production efficiency across a wide range of aquaculture species.

2.1.6 Benefits and drawbacks of using medicinal plants in aquafeeds

Using medicinal plants like *Aloe vera*, *Pimenta dioica* (allspice), *Moringa oleifera*, *Rosmarinus officinalis* (rosemary), *Allium sativum* (garlic), and *Zingiber officinale* (ginger) offers both benefits and drawbacks (van Hai, 2015). These medicinal plants have the potential to improve feed nutritional quality and feed utilization, control disease outbreaks, enhance health and resistance against pathogens, and act as anaesthetics and sex reversal agents in fish culture. Besides, medicinal plants sound to be more appropriate for African smallholder aquaculture farmers for many reasons: (1) they are inexpensive, (2) readily available, (3) simple to apply, (4), diverse in nature, and (5) more biodegradable. However, the drawbacks of using these medicinal plants are associated with lack of standardization whereby potency and composition can vary due to growing conditions, preparation methods, and plant parts used (leaf or roots). Limited scientific validation also forms part of the disadvantages of using medicinal plants; although traditional knowledge is strong, some claims lack rigorous scientific validation. For example, *Pimenta dioica*, is less studied compared to garlic or ginger. Additionally, possible side effects and toxicity can be a drawback. Overuse of incorrect use can cause harm (e.g., *Artemisia afra* can be toxic at high doses) (Mbokane and Moyo, 2018a). Overharvesting concerns are also characterized as one of the drawbacks in the sustainability of using medicinal plants due to increased demand which leads to it, especially in wild plants like *Artemisia afra*.

2.1.7 *Artemisia afra* as a medicinal plant of choice for this study

Artemisia afra, commonly known as “Wilde-als” is one of the many *Artemisia* species that has been acclaimed to manage several health conditions in humans (du Toit and van der Kooy, 2019) and some domesticated animals such as chickens (Ferreira, 2009) and dogs (Mukinda and Syce, 2007). In humans, *A. afra* has been used directly or as leaves to cure ailments such as coughs, colds, influenza, and malaria (du Toit

and van der Kooy, 2019). Besides extensive research on *A. afra* composition and its application in humans, little information exists regarding its application in aquaculture. The existing information has indicated that *A. afra* could be used as a feed supplement in aquaculture for various reasons. Mbokane and Moyo (2018a, 2020a) reported that supplementing *A. afra* leaf powder at 10% diet inclusion levels enhanced the innate immune response of *O. mossambicus* and *C. gariepinus*.

This study investigated the effect of 10% *A. afra*-based diet on reducing stress in Mozambique tilapia and the African Sharptooth catfish. The study attempts to determine the importance of the 10% inclusion level of *Artemisia afra* in enhancing growth and innate immunity without affecting the normal physiological functioning of the fish. The existing information on using *A. afra* as a feed supplement in aquaculture was outlined in Chapter 1. The information generated will contribute to the formulation of dietary supplements that can enhance immunity and prevent stress-related diseases on tilapia and catfish farms.

CHAPTER 3

**EFFECTS OF HANDLING STRESS
ON GROWTH PERFORMANCE AND
INNATE IMMUNITY OF
OREOCHROMIS MOSSAMBICUS
AND *CLARIAS GARIEPINUS* FED
ARTEMISIA AFRA-BASED DIETS**

CHAPTER 3: EFFECTS OF HANDLING STRESS ON GROWTH PERFORMANCE AND INNATE IMMUNITY OF *OREOCHROMIS MOSSAMBICUS* AND *CLARIAS GARIEPINUS* FED *ARTEMISIA AFRA*-BASED DIETS

3.1 INTRODUCTION

Mozambique tilapia (*Oreochromis mossambicus*) and the African sharptooth catfish (*Clarias gariepinus*) are the most cultured species in the Limpopo Province (Rapatsa and Moyo, 2019; Moyo and Rapatsa, 2021). These two species are subjected to handling at fish farms (Colt *et al.*, 2011; Manuel *et al.*, 2014). Fish farmers handle fish to monitor weight, age, size, and length and also to assess their health status. Excessive handling can lead to poor growth, poor reproductive efficiency, a decrease in stress resistance, immune function suppression, and eventually an increase in mortality (Gabriel *et al.*, 2011; Rehman *et al.*, 2017).

The response of a fish to handling time is species-dependent (Milla *et al.*, 2021; Kotowych *et al.*, 2023). This handling takes place outside the water. Handling in fish refers to any physical or environmental interaction with fish by humans (or machinery) during aquaculture or research operations that alters their normal behavior or physiology. In cichlidae, *Oreochromis* spp. maximum handling time was reported to be 20 minutes (Bernardino *et al.* 2020). Meanwhile, Foo and Lam (1993) reported 15 minutes as the maximum handling time for *O. mossambicus*. In the ictaluridae family, Davis *et al.* (2002) and Aguirre-Guzman *et al.* (2016) reported that the maximum handling time for *Ictalurus punctatus* was 45 minutes. Furthermore, Adeyemo *et al.* (2009) showed that the maximum handling time for *C. gariepinus* was 30 minutes. In relation to Salmonidae, Morales *et al.* (1990) indicated that the maximum handling time for *Onchorhynchus mykiss* was 5 minutes. Fish size can also have an impact on determining the appropriate handling time for fish at fish farms. According to Akinrotimi *et al.* (2011), the maximum handling time for *Tilapia guineensis* weighing 408.0 g was 20 minutes. Similarly, Abraham-Olukayode and Oramadike (2015) recorded a maximum handling time of 20 minutes in *T. guineensis* of similar size (390.5 g). Ide and Hoffman (2002) observed that the maximum handling time for *O. niloticus* weighing 114.6 g was 25 minutes. However, Shokr (2011) observed a maximum

handling time in *O. niloticus* weighing 40.0 g to be 15 minutes. Davis *et al.* (2002) recorded that the maximum handling time was 45 minutes in *I. punctatus* weighing 60.4 g. Aguirre-Guzman *et al.* (2016) also observed a 45-minute handling in *I. punctatus* of different sizes 200.0 g. Adeyemo *et al.* (2009) recorded the maximum handling time of 30 minutes in *C. gariepinus* weighing 585.0 g. However, Abdel-Hay *et al.* (2020) observed a maximum handling time of 25 minutes in *C. gariepinus* of different sizes (100.3 g).

Fish frequently indicate alterations in opercular rate as a physiological response to handling, primarily when handled in a stressful manner. Handling fish in a stressful manner refers to any handling technique or condition that causes excessive physical or psychological stress, leading to altered behavior and physiological responses; like an increased opercular beat rate (gill cover movement used for respiration) (Zhou *et al.*, 2022). Frequently, this elevated opercular rate is a temporary reaction to handling stress. Extended periods of handling can result in fatigue and exhaustion, which can affect the growth of the fish. In addition, fish can respond to stress physiologically. Physiological response in fish can influence plasma cortisol and blood chemistry. Fish often release elevated levels of cortisol, the primary stress hormone in fish, as well as in many other vertebrates, including humans. In addition to physiological responses, blood chemistry is also the most useful tool for assessing health status and monitoring stress in fish (Arfuso *et al.*, 2022). A decrease in blood parameters weakens the immune system, making fish more susceptible to diseases and infections, and this response compromises their growth.

There are few ways that can be used to reduce stress in aquaculture. One of the ways is to use medicinal plants. Some plants contain tryptophan (Ur Rashid *et al.*, 2019). It has been shown that the effect of handling times can be ameliorated if the fish are fed on a diet that contains active ingredients such as tryptophan (Awad and Awaad, 2017). Tryptophan is the essential dietary amino acid that acts as a precursor of serotonin (5-HT) and is known to affect food intake, aggression, stress, and cannibalism in vertebrates, including fish (Sahu *et al.*, 2020). Mbokane and Moyo (2018a; 2020a) reported that *A. afra* at a concentration of 10% boosted *O. mossambicus* and *C.*

gariiepinus immunity and enhanced disease resistance. However, despite the fact that *O. mossambicus* and *C. gariiepinus* are widely cultured species in Southern Africa, studies that have been carried out to investigate the effect of handling time on the growth performance and innate immunity using *A. afra* could not be found. The aim of this chapter was to explore how this plant might be used to reduce stress in tilapia and catfish culture.

3.2 SPECIFIC OBJECTIVES

The specific objectives of this chapter were to:

1. Determine the effect of *A. afra*-based diets on the opercular beat rate in *O. mossambicus* and *C. gariiepinus* when exposed to different handling times.
2. Determine the effect of *A. afra*-based diets on growth performance and feed utilization in *O. mossambicus* and *C. gariiepinus* when exposed to different handling times.
3. Determine the effect of *A. afra*-based diets on haematological and immunological parameters in *O. mossambicus* and *C. gariiepinus* when exposed to different handling times.

3.3 NULL HYPOTHESIS

The null hypothesis of this chapter was:

1. Handling stress does not affect the opercular beat rate of *O. mossambicus* and *C. gariiepinus* fed with the *A. afra*-based diets.
2. Handling stress does not affect the growth performance and feed utilization of *O. mossambicus* and *C. gariiepinus* fed with the *A. afra*-based diets.
3. Handling stress does not affect the haematological and innate immunity of *O. mossambicus* and *C. gariiepinus* fed with the *A. afra*-based diets.

3.4 MATERIALS AND METHODS

3.4.1 Fish acclimatisation

Oreochromis mossambicus and *Clarias gariepinus* were obtained from the Aquaculture Research Unit, University of Limpopo, South Africa. The experiment commenced on the 08th May 2023 until 05th April 2023. Fish were stocked in triplicate tanks at 15 fish per tank and were randomly kept in the experimental tanks, where they were acclimatised for two weeks before the experiment. The weighing of the fish took place after the acclimatisation period was over. Fish were fed a maintenance diet consisting of a normal commercial tilapia diet with a 34% protein content, 3% lipid, and a gross energy content of 17 MJ/kg dry matter. Before the experiment, randomly selected from the group of fish to be used in the experiments were examined for the presence of diseases and parasites.

3.4.2 Feed preparation

The *Artemisia afra* essential oil was purchased from a local farm around the Tzaneen area, Limpopo Province, South Africa. Two dosage levels were prepared by adding *A. afra* to dry ingredients at concentrations of 0% and 10% *A. afra*, denoted as D1 and D2, respectively. The *A. afra* diets were prepared by mixing fish meal, canola meal, sunflower meal, maize gluten, wheat middling, and maize meal as indicated in Table 3.1. The diets were formulated using the Winfeed 3, EFG Software (Natal) program. To ensure a homogenized mixture, dry ingredients were mixed for 30 minutes in a Hobart mixer (Troy, Ohio, USA). The *A. afra* oil was added and blending continued for 15 minutes. A 10-20% v/w of water was added to the mixture until a dough was formed. The mixture was extruded through a Hobart Pelletizer equipped with 2-3 mm holes to produce strings to attain pellets of a desired size. Pellets were dried in the sun; after drying them, they were packed in labelled polyethylene buckets, closed, and stored at -4°C until needed. The experimental diets were isoenergetic and were formulated to be nutritionally identical (40.68% protein and 16.1 MJ Kg⁻¹).

3.4.3 Proximate analysis

Based on the guidelines stipulated by the Association of Official Analytical Chemists (AOAC International, 2012), the diets were analysed for dry weight, crude protein, crude lipid, crude fibre, and gross energy. A triplicate analysis of each diet sample was performed. In order to determine gross energy, an automatic adiabatic bomb calorimeter was applied (Gallenkamp and Co Ltd, England). An increase in the temperature of the water around a burning bomb was used to measure the heat resulting from the burning of the sample. As a standard, benzoic acid was utilized. An energy value from a known standard was used to calibrate the system. The energy content in each sample was calculated according to the formula:

Gross Energy (kJ/g) = [(final temperature-initial temperature) x 10.82]-0.0896 / sample weight (g). where: 10.82 = Heat capacity of the calorimeter (kJ/K) 0.0896 = Combined energy value of nickel wire and cotton (kJ/g).

Dry matter was expressed as a percentage of the initial sample weight. Moisture content was determined by drying samples to constant weight in an oven (Gallenkamp, UK) at 105°C for 12hrs. Crude protein (CP) was calculated from the nitrogen content of each sample, and it was determined using the Kjeldahl method. Samples were digested in concentrated sulphuric acid using a Digestor 2040 (FOSS, Denmark) followed by distillation using a Kjeltac 2300 auto-analyser (FOSS, Denmark) to determine nitrogen content, which was converted to crude protein using a conversion factor of 6.25. Lipid content was assessed using the solvent extraction method that uses a Soxhlet extraction unit (Tecator Soxtech HT 1043 Extraction unit). Crude lipid in diets and ingredients was determined with petroleum ether at 50°C. This method depends upon the heating of solvent, which is allowed to pass through the sample to extract the lipid. The extract is collected and when the process is completed the solvent is evaporated and the remaining crude lipid is dried and weighed. Crude lipid was then calculated as Crude lipid (%) = (extracted lipid/sample weight) x 100. The crude fibre was determined as loss in weight after ignition of dried lipid-free residues after digestion with 1.25% sulphuric acid solution and 1.25% sodium hydroxide solution. This method depends upon the digestion of moisture-free and solvent extracted samples with a weak acid solution and then with a weak base solution. The remaining residue was then ashed for 2 hours at 550°C in a muffle furnace, and the difference in weight on ashing was regarded as the crude fibre (hydrolysis-resistant organic matter).

The extracted fibre was then expressed as a percentage of the original un-defatted sample and calculated as Fibre (%) = [(digested sample – ashed sample) / sample weight] x 100.

Table 3. 1: Feed ingredient (g kg⁻¹) and proximate composition of the experimental diets.

Ingredients	Diets	
	D1 (0%)	D2 (10%)
Fishmeal	10	10
Soybean meal	7	7
Canola meal	16	16
Sunflower meal	14	14
Maize gluten	11	11
Cellulose	10	0
Wheat middling	2	2
Maize meal	24	24
Canola oil	2	2
Vitamin/Mineral premix	2	2
Binder	2	2
<i>Artemisia afra</i> (g kg ⁻¹)	0	10
Moisture	8.05	9.04
Crude protein	40.11	40.68
Crude lipid	8.06	8.07
Ash	6.90	7.90
Gross energy (MJ kg ⁻¹)	15.8	16.1

Vitamin/mineral premix: Vitamin A, 4500, I. U. Vitamin D, 11252.U; vitamin E. 71.U; vitamin K3, 2mg; Vitamin B12 0.015mg, pantothenic acid; 5mg, nicotinic acid, 14mg; folic acid, 0.4mg; biotin, 0.04mg; choline, 150mg, cobalt 0.2mg; copper 4.5mg; iron 21mg; manganese 20mg; iodine, 0.6mg; selenium 2.2mg; zinc 20mg, antioxidant, 2mg.

3.4.4 Experimental system

The experiment was conducted in rectangular concrete tanks connected to an independently controlled recirculating system in the solar-heated tunnel. Three systems were used in this experiment. The system consists of twenty-one rectangular self-cleaning concrete tanks (1000 L) (Figure 3.1). Aged municipal water was pumped from reservoir through two sand-filters to an elevated 5000 L Jojo tank. From there, the water was provided to each at a rate of 1 L per minute. Each tank was filled with aged water up to the 900 L mark. The recirculating system consists of filtration, aeration, and heating components. The standpipe was installed at the bottom of each tank to transfer water from the tank into a 40 mm PVC pipe that flowed to the reservoir. The sand filters were back washed once a week to remove accumulated sludge from uneaten food and faeces, which clogs the filters if allowed to build up over a long time. This was done to guarantee that the water supply to the tanks was not disrupted or slowed down by blocked filters.



Figure 3. 1: The rectangular concrete tanks connected to a recirculating system at the Aquaculture Research Unit, University of Limpopo, South Africa.

3.4.5 Experimental design

Oreochromis mossambicus (360) with an average mean weight of 30.65 ± 4.61 g and *Clarias gariepinus* (360) with an average mean weight of 38.36 ± 4.89 g were stocked in 48 tanks (24 tanks per fish species). The experiment was replicated three times. All fish were hand-fed their specified diets until apparent satiation (until one or two pellets remained un-eaten for two minutes) at 09:00 and 15:00 hours for 45 days. There were occasional refusals, indicated by the presence of uneaten pellets. These were minimal (one or two pellets) and used as the benchmark to determine satiation. The uneaten feed was not force-fed or reintroduced; instead, feeding was stopped once refusal was observed. The amount of feed consumed was recorded for each treatment. Every two days, the water quality was monitored to make sure that environmental conditions were suitable for *O. mossambicus* and *C. gariepinus*. Measured physicochemical parameters included water temperature, dissolved oxygen, pH, and conductivity. During the experiment, the average values of these water quality parameters were as follows: water temperature ranged from 26.4 °C to 29.5 °C (average 28 ± 0.2), pH (7.5 ± 0.2), dissolved oxygen (6.79 ± 0.2) mg L⁻¹, and conductivity (590) µS. In this study, ambient or tunnel temperature were not recorded.

3.4.6 The effect of handling times in *O. mossambicus* and *C. gariepinus*

The experiment on *O. mossambicus* consisted of four handling times per dietary treatment, namely control (0), 10, 15, and 20 minutes. Control denotes that there was no fish handling. All fish were scooped out of the tank and were handled by gripping the whole body twice daily (09:00hr and 15:00hr) for 3 days a week (Monday, Wednesday, and Friday). Immediately after handling, fish were taken back into the experimental tank while one was placed in a separate small glass tank (10 L) to count opercular beats per minute twice daily (09:00hr and 15:00hr). *Clarias gariepinus* experiment also consisted of four handling times per dietary treatment. The same handling procedure was used, although the handling times used were different, namely control (0), 15, 30, and 45 minutes. Control also denoted that there was no handling of fish. Different handling times were used because fish species used in this experiment are different and respond differently to handling. Fish were handled using a standardized procedure which included netting and keeping them out of water for

the full duration of the treatment time. Control fish were not removed from the water at all. Thus, handling time directly equates to out-of-water time.

After the fish were handled, they were monitored daily for any clinical signs, abnormal behaviour, or mortalities from the handling effect. Throughout the acclimatization period and handling test, environmental conditions were maintained as optimum as possible for *O. mossambicus* and *C. gariepinus*. Growth parameters, haematological and immunological parameters were measured at the end of the experiment. All ethical protocols were observed and approved by the University of Limpopo Animal Research Ethical Committee (AREC/24/2023: PG).

3.4.7 Determination of growth performance indices

All fish were sampled from the tanks and weighed individually at the culmination of the feeding trial of 45 days to determine the growth performance indicators for handling times. At the end of the experimental period, fish were individually weighed, and the following growth performance indices were calculated:

Specific growth rate (SGR) was calculated according to Winberg (1956) as:

$$\text{SGR} = [(\ln \text{ final weight} - \ln \text{ initial weight})/\text{time}] \times 100.$$

Feed utilisation was determined using **feed conversion ratio** and it was calculated as:

$$\text{FCR} = \text{Food consumed (g)}/\text{Weight gain (g)},$$

$$\text{Feed intake (FI, g fish}^{-1} \text{ 45 days}^{-1}) = \text{Dry feed intake (g)}/\text{number of fish/days}.$$

3.4.8 Haematological analyses

At the end of the experimental period, five fish from each treatment were anesthetized with 2-phenoxyethanol (1ml L⁻¹). Blood samples were collected from the fish through the caudal vein using a 5 ml sterile syringe. For haematological parameters, blood was kept in heparinized tubes. The haematological variables measured were white blood cell count (WBC x 10³/L), haemoglobin concentration (Hgb, g/dL), haematocrit (Hct,

%), red blood cell count (RBC, $\times 10^6/\text{L}$), platelet count ($\times 10^6/\text{L}$), mean corpuscular volume (MCV, fL), mean corpuscular haemoglobin (MCH, g/dL), and mean corpuscular haemoglobin concentration (MCHC, g/dL). Blood performance was calculated as follows:

Blood performance = $\text{Ln} [\text{Hb}(\text{g/dL})] + \text{Ln} [\text{HCT} (\%)] + \text{Ln} \text{RBC} (\times 10^6/\text{L}) + \text{Ln} \text{WBC} (\times 10^3/\text{L})$ according to Esmaeili (2021).

Where the Hb is the haemoglobin (g/dL), HCT is the haematocrit (%), RBC is the red blood cells ($\times 10^6/\text{L}$) and WBC as white blood cells ($\times 10^3/\text{L}$). It is necessary to measure both WBC, RBC, HCT, and HGB for Blood Performance calculations.

3.4.9 General immune parameters

Lysozyme activity was also assessed using the turbidimetric assay (Ellis, 1999) at the end of the experimental period. A suspension of 875 μL of *Micrococcus lysodeikticus* (Sigma, ATCC 4698) at a concentration of 0.2 mg mL^{-1} (in PBS) and was added to 25 μL of serum samples were measured spectrophotometrically at 530 nm after 0.5 and 4.5 min at 25°C , using a spectrophotometer. A unit of lysozyme activity was defined as the amount of serum causing a reduction in absorbance of 0.001 min^{-1} .

At the end of the experimental period, plasma cortisol level was determined using a monoclonal antibody enzyme-linked immunosorbent assay (ELISA) quantification kit (Enzo Life Sciences, Inc., NY, USA). Thawed plasma and standards were pipetted into a 96-well microplate. All solutions were prepared according to the manufacturer's instructions. The assay validity for fish cortisol has been reported to be comparable to Cayman Cortisol Assay Kit (Thompson *et al.*, 2014). Cortisol standards were used to generate the standard curve. Plasma cortisol levels were measured at an optical density of 405 nm using Halo MPR-96 Visible Microplate Reader (Dynamica GmbH, UK) by comparing them with a range of standard concentrations. The measurements were performed in triplicate, being expressed as $\mu\text{g mL}^{-1}$.

3.4.10 Statistical analysis

Percentage data was arcsine transformed prior to carrying out regression analysis. The regressions were significant when $P < 0.05$. Analysis of covariance (ANCOVA) was used to test if there were significant differences between D1 (0% *A. afra*) and D2 (10% *A. afra*) regressions for opercular beat rate, specific growth rate, feed intake, and feed conversion ratio. The relationship between covariate (handling levels) and dependent variables (OBR, SGR, FCR, FI, and BP) was examined using a linear regression model ($y = aX + b$). Wherein “a” is the slope and “b” is the intercept. The slope represents the relationship between the covariate and dependent variables. Normality and homogeneity of variance was confirmed using the Shapiro-Wilk normality test and Levene test, respectively using R studio (version 4.3, 2023). Analysis of covariance (ANCOVA) was also used on haematological parameters (WBC, RBC, HGB, HCT, MCV, MCH, MCHC, and Platelets) and immunological parameters (lysozyme activity, and plasma cortisol) fed with the 0% and 10% *A. afra*-based diets.

3.5 RESULTS

3.5.1 Effect of handling times on opercular beat rate

The opercular beat rate in *O. mossambicus* fed D1 ($R^2 = 0.824$) and D2 ($R^2 = 0.921$) increased with an increase in handling times (Figure 3.2A). In fish fed with D1 and D2, fish handled for 20 minutes recorded the highest opercular beat rate. However, there were no significant differences ($P > 0.05$, ANCOVA) among handling times in fish fed the D1. Opercular beat rate was significantly higher ($P < 0.05$, ANCOVA) in fish fed D1 than in fish fed D2 (Figure 3.2A).

Increasing handling times had a significant ($P < 0.05$) positive linear relationship with opercular beat rate in *C. gariepinus* fed with D1 ($R^2 = 0.974$) and D2 ($R^2 = 0.900$) (Figure 3.2B). The highest OBR was recorded in fish handled for 45 minutes, fed with D1 and D2. The opercular beat rate was significantly higher ($P < 0.05$, ANCOVA) in fish fed with D1 than in fish fed with D2 (Figure 3.2B).

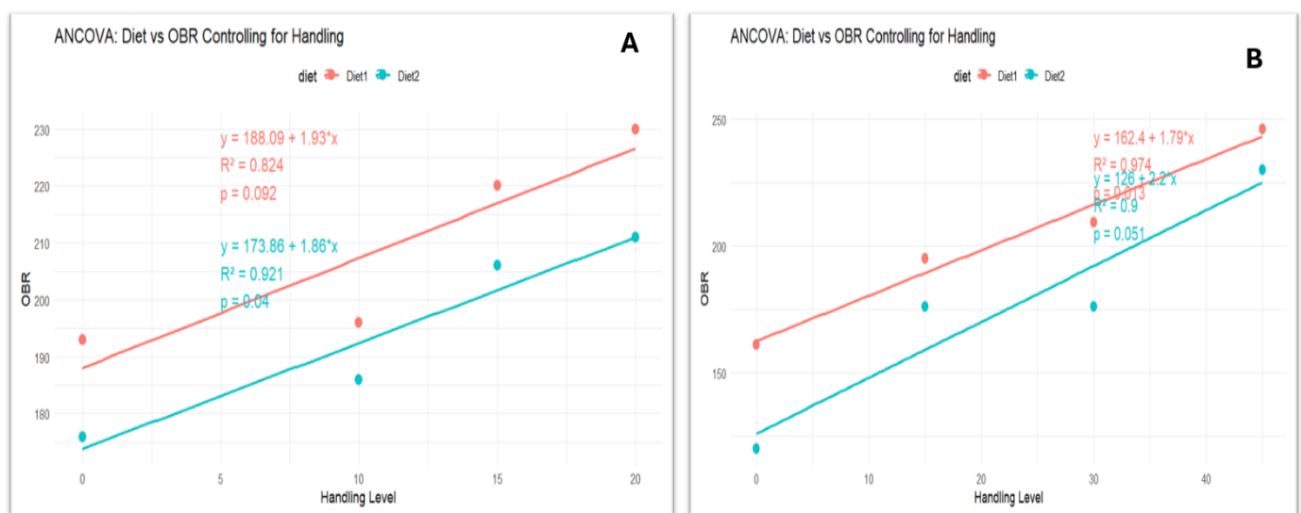


Figure 3. 2: Linear regression of handling times on opercular beat rate of *O. mossambicus* (A) and *C. gariepinus* (B) fed D1 and D2.

3.5.2 Effect of handling times on growth performance and feed utilization

3.5.2.1 Specific growth rate

In *O. mossambicus* fed both D1 and D2, SGR was highest in control. Specific growth rate was higher ($P < 0.05$) in fish fed D2 than in fish fed D1 (Figure 3.2A). Both D1 and

D2 showed negative linear relationships across handling times. However, in fish fed D1, there were no significant differences ($P > 0.05$, ANCOVA) among handling times.

Similarly, both D1 and D2 showed a negative linear relationship across handling times (Figure 3.3B). Specific growth rate followed the same trend as the highest SGR was recorded in the control. No significant differences ($P > 0.05$) were observed in *C. gariepinus* fed D1 and D2. Fish fed D2 showed a higher ($P < 0.05$) SGR than in fish fed D1 (Figure 3.3B).

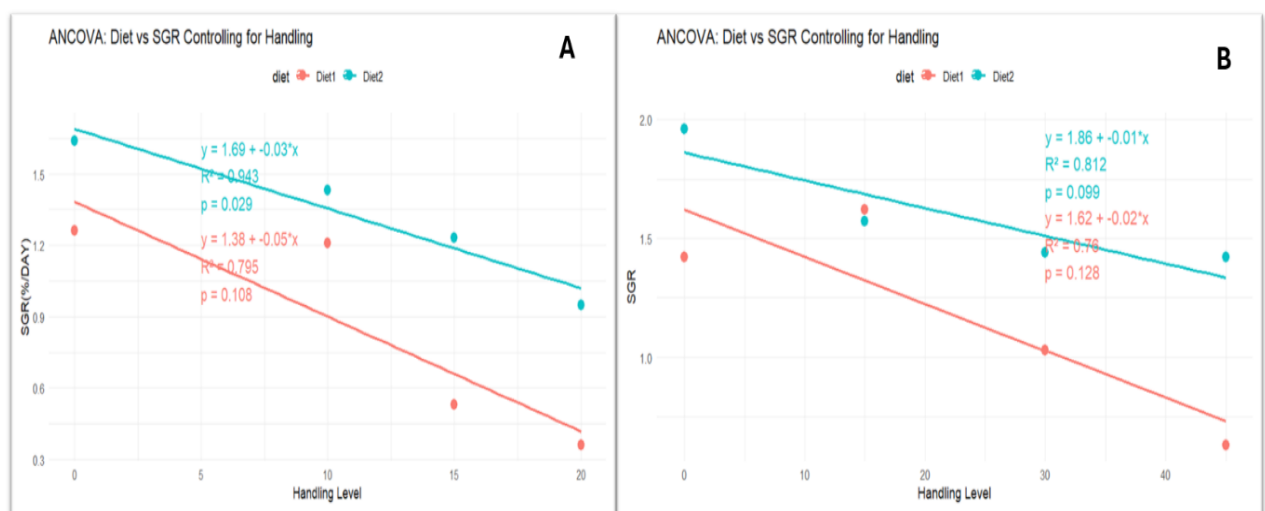


Figure 3. 3: Linear regression of handling times on specific growth rate of *O. mossambicus* (A) and *C. gariepinus* (B) fed D1 and D2.

3.5.2.2 Feed conversion ratio

There were positive linear relationships ($P < 0.05$, ANCOVA) between handling times and FCR in fish fed D1 ($R^2 = 0.961$) and D2 ($R^2 = 0.915$) (Figure 3.4A). The best FCR in *O. mossambicus* fed D1 was recorded in control. Fish fed D2 also followed the same trend. Feed conversion ratio was much higher ($P < 0.05$) in fish fed D1 than in fish fed D2 (Figure 3.4A).

The best FCR was similarly noted in control when *C. gariepinus* was fed D1 and D2 (Figure 3.4B), and there were significant differences ($P < 0.05$, ANCOVA) across

handling times. Increasing handling times in *C. gariepinus* fed D1 ($R^2 = 0.966$) and D2 ($R^2 = 0.986$) had a positive linear relationship ($P < 0.05$) with FCR. Similar to *O. mossambicus*, fish fed D1 recorded a higher FCR than in fish fed D2 (Figure 3.4B).

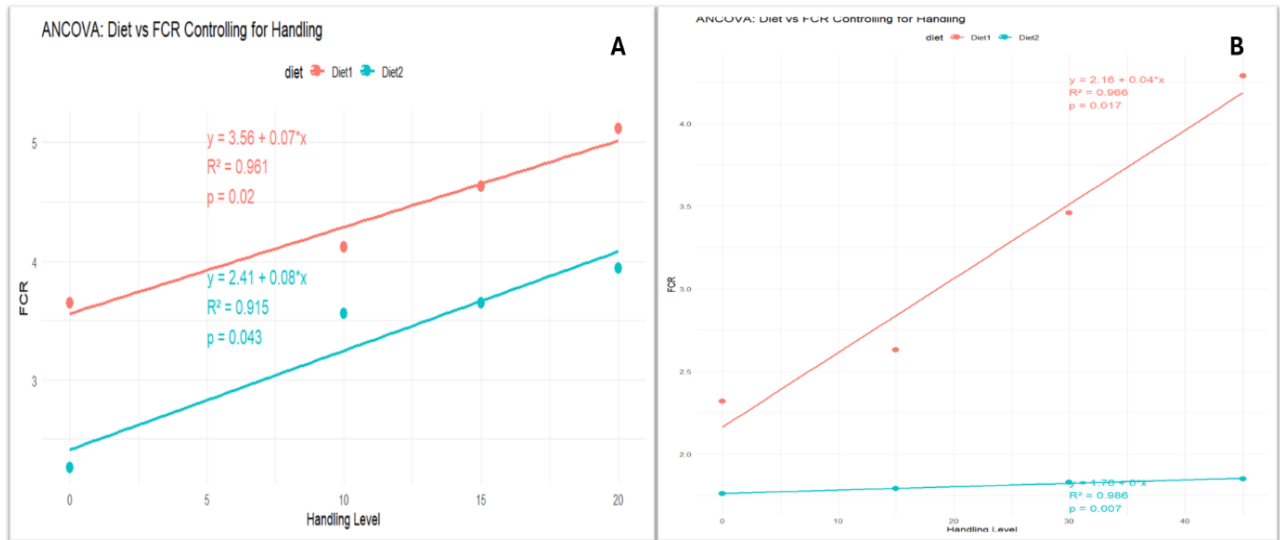


Figure 3. 4: Linear regression of handling times on feed conversion ratio of *O. mossambicus* (A) and *C. gariepinus* (B) fed D1 and D2.

3.5.2.3 Feed intake

Feed intake in *O. mossambicus* fed D1 decreased with an increase in handling times ($R^2 = 0.911$) (Figure 3.5A). Fish fed D2 ($R^2 = 0.759$) also followed the same trend. Feed intake in fish fed D1 and D2 was highest in control. Both D1 and D2 showed a negative linear relationship throughout handling times. However, no significant differences ($P > 0.05$, ANCOVA) were observed among handling times in fish fed D2. Similar to SGR, the highest feed intake was recorded in D2 than in D1 (Figure 3.5A).

In *C. gariepinus* fed D1, a decrease in feed intake was similarly observed with an increase in handling times ($R^2 = 0.971$) (Figure 3.5B). A similar trend was observed in D2 ($R^2 = 0.957$) (Figure 3.5B). Fish in control recorded the highest feed intake, and it was significantly different ($P < 0.05$, ANCOVA) across handling times in fish fed both the D1 and D2. Feed intake was lower ($P < 0.05$) in fish fed D1 than in fish fed D2 (Figure 3.5B).

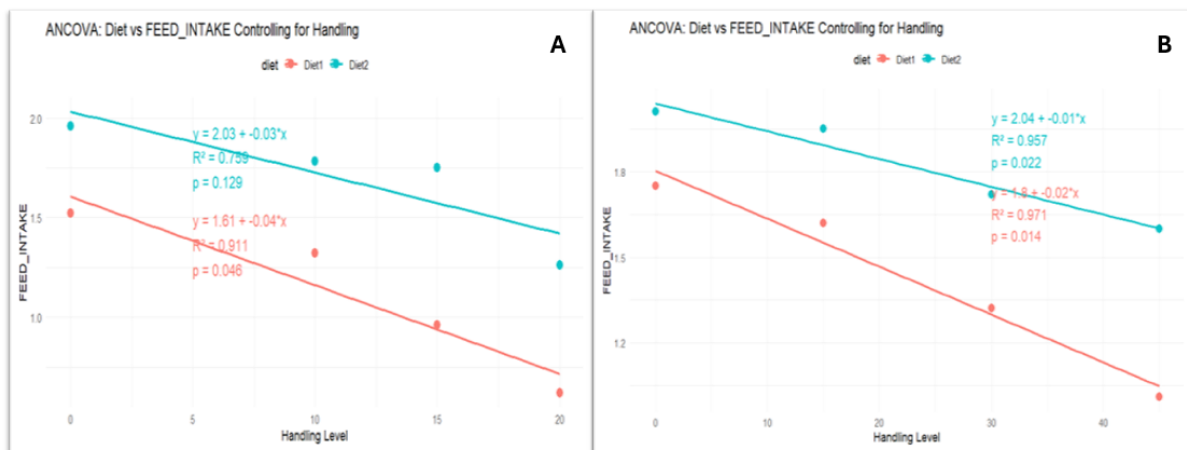


Figure 3. 5: Linear regression of handling times on feed intake of *O. mossambicus* (A) and *C. gariepinus* (B) fed D1 and D2.

3.5.2.4 Survival rate

Survival rate slopes were much higher ($P < 0.05$, ANCOVA) in fish fed D2 than in fish fed D1 in both fish species (Figure 3.6A and 3.6B). The survival rate in *O. mossambicus* and *C. gariepinus* fed D1 decreased significantly ($P < 0.05$) with increasing handling times (Figure 3.6A and 3.6B). No mortality was observed in both *O. mossambicus* and *C. gariepinus* fed D2, and no significant differences ($P > 0.05$, ANCOVA) across handling times were observed (Figure 3.6A and 3.6B).

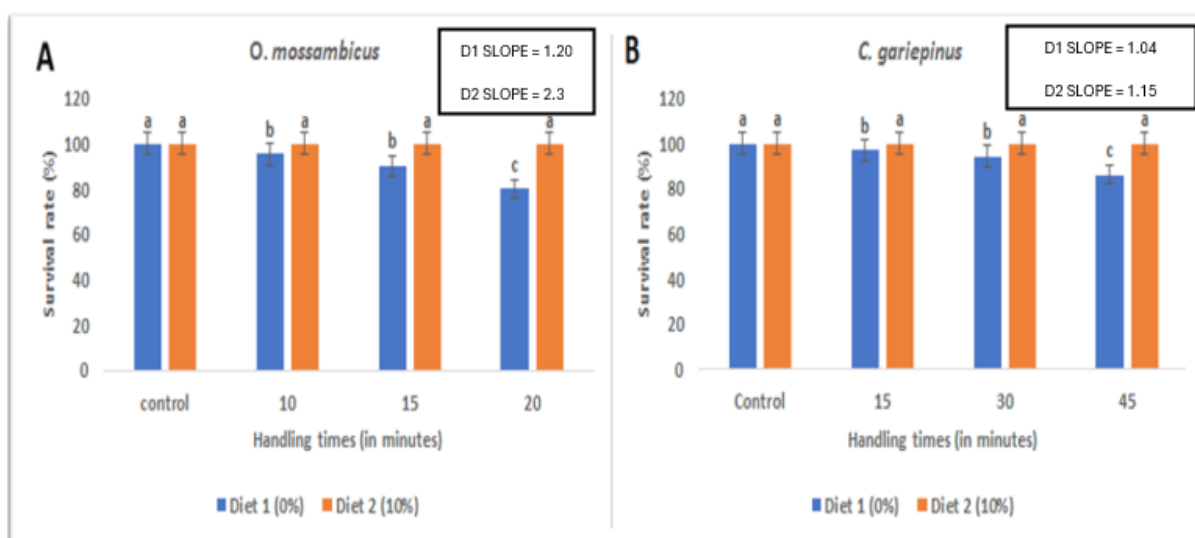


Figure 3. 6: The effect of different handling times on survival rate (%) of *O. mossambicus* (A) and *C. gariepinus* (B) fed with the 0% *A. afra*-based diet (D1) and

10% *A. afra*-based diet (D2). Bars with different letters differ significantly ($P < 0.05$, ANCOVA).

3.5.3 Effect of handling times on haematological parameters

In *O. mossambicus* fed D1, WBC, and RBC decreased with increasing handling times (Table 3.2). The highest WBC ($4.45 \pm 0.26 \times 10^3/L$) and RBC ($1.65 \pm 0.01 \times 10^6/L$) were observed in control. However, there were no significant differences ($P > 0.05$) in WBC and RBC among handling times. The HCT and HGB counts decreased with increasing handling times (Table 3.2). The HCT ($25.49 \pm 2.15\%$) and HGB (8.26 ± 1.25 g/dL) in control were significantly higher ($P < 0.05$) than fish handled for 15 and 20 minutes. The HCT and HGB did not differ significantly ($P > 0.05$) with fish handled for 10 minutes. MCV, MCH, and MCHC decreased with increasing handling times (Table 5.2). However, no significant differences ($P > 0.05$) in MCV, MCH, and MCHC were observed among handling times. Platelets had no discernible pattern with increasing handling times (Table 5.2). The highest platelet count was observed in fish handled for 10 minutes ($65.44 \pm 3.54 \times 10^6/L$), and no significant differences ($P > 0.05$) were observed across handling times.

In *O. mossambicus* fed D2, WBC increased with increasing handling times (Table 3.2). The highest WBC count was observed in fish handled for 20 minutes ($6.68 \pm 0.14 \times 10^3/L$), and there were no significant differences ($P > 0.05$) across handling times. A similar trend was observed in Haemoglobin counts, MCH, MCHC, and platelet counts. The increase in handling times led to a decrease in red blood cell counts and haematocrit counts (Table 3.2). Fish in control recorded the highest RBC and HCT levels and were observed to be $1.85 \pm 0.46 \times 10^6/L$ and $27.35 \pm 0.78 \%$, respectively. The differences between handling times were not statistically significant ($P > 0.05$, ANCOVA). MCV recorded a similar trend as RBC and HCT (Table 3.2). Fish fed D2 had higher ($P < 0.05$, ANCOVA) WBC, RBC HCT, HGB, MCV, MCH, MCHC and platelets slopes than in fish fed D1.

Clarias gariepinus fed D1 showed a similar trend to *O. mossambicus* WBC and RBC fed D1 (Table 3.3). The highest WBC and RBC were also observed in the control

group. Haematocrit and haemoglobin count also decreased with increasing handling times (Table 3.3). The HCT and HGB in control were significantly higher ($P < 0.05$) than fish handled for 15 and 20 minutes. The HCT and HGB also did not differ significantly ($P > 0.05$) with fish handled for 10 minutes. The MCV, MCH, MCHC, and platelets followed the same pattern as WBC and RBC (Table 3.3).

In *C. gariepinus* fed D2, WBC also increased with an increase in handling times (Table 3.3). Fish handled for 45 minutes recorded a higher WBC ($5.89 \pm 1.47 \times 10^3/L$), and it was not significantly different ($P > 0.05$, ANCOVA) from other handling times. HGB, MCV, and platelets also increased with an increase in handling times, and they were not significantly different ($P > 0.05$, ANCOVA) from other handling times. Red blood cells and HCT decreased with an increase in handling times. Red blood cells and HCT in *O. mossambicus* fed D2 showed a similar pattern to that of *C. gariepinus* fed D2. An increase in handling times led to no noticeable pattern in MCH and MCHC. *Clarias gariepinus* also had similar slope pattern as *O. mossambicus*. However, RBC slopes were similar in both D1 and D2 (Table 3.3).

Table 3. 2: Effect of handling times on the haematological parameters of *O. mossambicus* fed D1 (0% *A. afra*-based diet) and D2 (10% *A. afra*-based diet) based diets after 45 days of feeding (n=9). Values are the means of three replicates \pm SD.

Haematological parameters	Handling times							
	D1 (0%)				D2 (10%)			
	Control	10 minutes	15 minutes	20 minutes	Control	10 minutes	15 minutes	20 minutes
WBCs ($10^3/L$)	4.45 \pm 0.26 ^a	3.69 \pm 0.14 ^a	3.21 \pm 0.25 ^a	2.14 \pm 0.42 ^a	5.49 \pm 0.36 ^a	6.03 \pm 0.28 ^a	6.53 \pm 0.29 ^a	6.68 \pm 0.14 ^a
RBCs ($10^6/L$)	1.65 \pm 0.01 ^a	1.21 \pm 0.06 ^a	0.94 \pm 0.02 ^a	0.63 \pm 0.08 ^a	1.85 \pm 0.46 ^a	1.83 \pm 0.17 ^a	1.82 \pm 0.26 ^a	1.80 \pm 0.20 ^a
HCT (%)	25.49 \pm 2.15 ^a	22.14 \pm 1.26 ^a	16.47 \pm 3.45 ^b	11.46 \pm 1.32 ^b	27.35 \pm 0.78 ^a	26.99 \pm 0.47 ^a	26.06 \pm 0.59 ^a	25.16 \pm 1.49 ^a
HGB (g/dL)	8.26 \pm 1.25 ^a	7.96 \pm 1.05 ^a	5.21 \pm 0.36 ^b	5.11 \pm 0.25 ^b	11.81 \pm 0.60 ^a	11.92 \pm 1.93 ^a	12.47 \pm 0.87 ^a	12.96 \pm 1.98 ^a
MCV (fL)	100.26 \pm 1.21 ^a	91.45 \pm 5.14 ^a	85.24 \pm 1.36 ^a	80.36 \pm 2.14 ^a	112.1 \pm 1.78 ^a	108.72 \pm 1.75 ^a	108.66 \pm 3.98 ^a	104.90 \pm 2.97 ^a
MCH (pg)	50.32 \pm 2.14 ^a	42.14 \pm 3.42 ^a	41.14 \pm 2.26 ^a	40.36 \pm 3.24 ^a	68.92 \pm 0.48 ^a	69.57 \pm 1.16 ^a	70.16 \pm 0.07 ^a	71.22 \pm 1.34 ^a
MCHC (g/dL)	53.45 \pm 2.56 ^a	51.36 \pm 6.54 ^a	51.32 \pm 2.69 ^a	50.41 \pm 3.95 ^a	54.93 \pm 0.50 ^a	55.13 \pm 0.75 ^a	55.63 \pm 2.38 ^a	55.65 \pm 0.54 ^a
Platelets ($10^6/L$)	65.25 \pm 4.65 ^a	65.44 \pm 3.54 ^a	63.21 \pm 8.52 ^a	62.25 \pm 5.22 ^a	66.75 \pm 1.17 ^a	66.80 \pm 0.57 ^a	67.97 \pm 0.44 ^a	68.98 \pm 0.42 ^a

In each row, different letters indicate significant differences between groups ($P < 0.05$, ANCOVA) RBC: red blood cell counts; HCT: haematocrit; HGB: haemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; WBC: white blood cell counts. Values are presented as means \pm standard deviation.

Table 3. 3: Effect of handling times on the haematological parameters of *C. gariepinus* fed D1 (0% *A. afra*-based diet) and D2 (10% *A. afra*-based diet) based diets after 45 days of feeding (n=9). Values are the means of three replicates \pm SD.

Haematological parameters	Handling times							
	D1 (0%)				D2 (10%)			
	Control	15 minutes	30 minutes	45 minutes	Control	15 minutes	30 minutes	45 minutes
WBCs ($10^3/L$)	4.15 \pm 1.26 ^a	4.05 \pm 0.65 ^a	3.91 \pm 0.22 ^a	3.85 \pm 1.65 ^a	4.69 \pm 0.53 ^a	5.72 \pm 0.44 ^a	5.86 \pm 1.72 ^a	5.89 \pm 1.47 ^a
RBCs ($10^6/L$)	2.14 \pm 0.34 ^a	2.11 \pm 0.31 ^a	1.86 \pm 0.21 ^a	1.82 \pm 0.36 ^a	2.59 \pm 1.69 ^a	2.49 \pm 1.59 ^a	2.42 \pm 1.67 ^a	2.39 \pm 1.28 ^a
HCT (%)	25.14 \pm 2.56 ^a	24.26 \pm 6.24 ^a	13.33 \pm 4.10 ^b	10.14 \pm 2.22 ^b	27.26 \pm 1.24 ^a	27.19 \pm 1.89 ^a	27.16 \pm 1.06 ^a	27.11 \pm 1.59 ^a
HGB (g/dL)	16.21 \pm 1.24 ^a	14.25 \pm 0.34 ^a	7.45 \pm 4.26 ^b	6.22 \pm 2.4 ^b	18.36 \pm 2.13 ^a	18.49 \pm 2.59 ^a	18.52 \pm 2.30 ^a	18.55 \pm 2.09 ^a
MCV (fL)	82.24 \pm 6.45 ^a	81.30 \pm 4.21 ^a	80.85 \pm 3.25 ^a	80.11 \pm 1.53 ^a	110.00 \pm 1.32 ^a	114.72 \pm 1.29 ^a	114.72 \pm 1.53 ^a	114.96 \pm 1.44 ^a
MCH (pg)	69.24 \pm 8.26 ^a	65.22 \pm 2.14 ^a	62.11 \pm 4.21 ^a	60.23 \pm 4.32 ^a	76.16 \pm 1.47 ^a	77.14 \pm 1.78 ^a	75.36 \pm 1.26 ^a	77.14 \pm 1.09 ^a
MCHC (g/dL)	50.33 \pm 14.1 ^a	48.52 \pm 3.12 ^a	45.12 \pm 2.26 ^a	43.25 \pm 3.21 ^a	56.16 \pm 1.32 ^a	55.95 \pm 1.24 ^a	56.47 \pm 1.13 ^a	56.96 \pm 3.36 ^a
Platelets ($10^6/L$)	80.26 \pm 3.21 ^a	79.15 \pm 1.36 ^a	77.23 \pm 1.25 ^a	75.26 \pm 3.25 ^a	73.16 \pm 2.06 ^a	73.99 \pm 2.11 ^a	74.44 \pm 2.89 ^a	75.56 \pm 5.36 ^a

In each row, different letters indicate significant differences between groups ($P < 0.05$, ANCOVA) RBC: red blood cell counts; HCT: haematocrit; HGB: haemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; WBC: white blood cell counts. Values are presented as means \pm standard deviation.

3.5.3.1 Effect of handling times on blood performance

Blood performance in *O. mossambicus* fed D1 decreased with an increase in handling times ($R^2 = 0.73$) (Figure 3.7A). The highest blood performance in *O. mossambicus* fed D1 was recorded in control. Contrary to fish fed D1, blood performance in fish fed D2 increased with an increase in handling times ($R^2 = 0.661$) (Figure 3.7A). However, the highest blood performance was recorded in fish handled for 20 minutes. No significant differences ($P > 0.05$) were observed across handling treatments in both D1 and D2. Blood performance was higher in fish fed D2 than in fish fed D1 (Figure 3.7A).

Clarias gariepinus blood performance showed a similar trend to *O. mossambicus* fed D1 and D2 (Figure 3.7B). However, fish fed D1 showed significant differences ($P < 0.05$) across handling treatments. Blood performance was higher in fish fed D2 than in fish fed D1 across the handling times (Figure 3.7B).

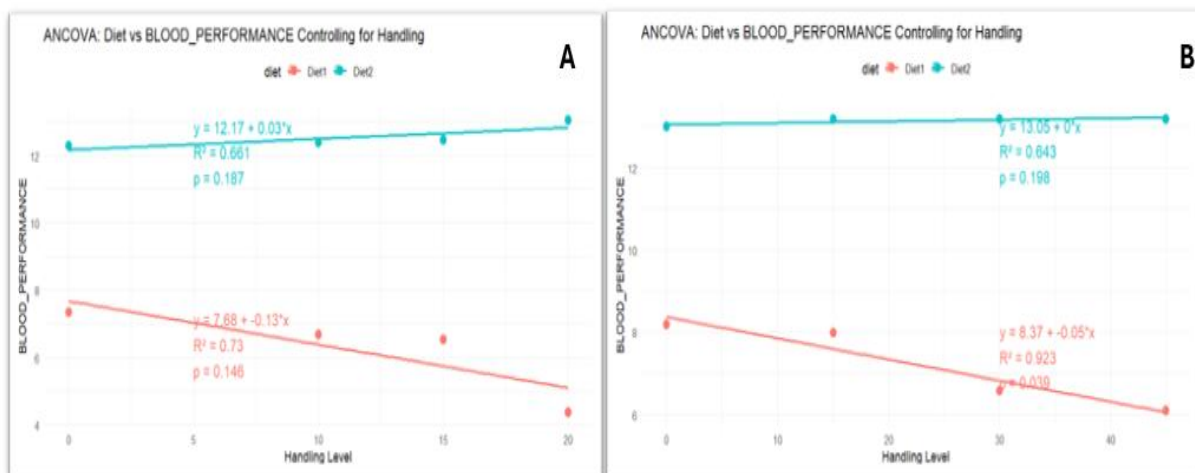


Figure 3. 7: Linear regression of handling times on blood performance of *O. mossambicus* (A) and *C. gariepinus* (B) fed D1 and D2.

3.5.4 Effect of handling times on immunological parameters

3.5.4.1 Lysozyme activity

Oreochromis mossambicus fed D2 recorded the highest ($P < 0.05$, ANCOVA) lysozyme activity values (1.60) than in fish fed D1 (1.54). An increase in handling times

led to a decline in lysozyme activity in *O. mossambicus* fed D1 (Figure 3.8A). Fish handled for 20 minutes ($1.01 \pm 0.06 \text{ mL}^{-1}$) recorded the lowest lysozyme activity, and it was significantly different ($P < 0.05$) from control and fish handled for 10 minutes. However, no significant differences ($P > 0.05$) in lysozyme activity were observed between fish handled for 15 minutes and 20 minutes. In *O. mossambicus* fed D2, an increase in handling times led to an increase in lysozyme activity (Figure 3.8A). However, there were no significant differences ($P > 0.05$) in lysozyme activity between handling times. The highest lysozyme activity was observed in fish handled for 20 minutes ($2.02 \pm 0.14 \text{ mL}^{-1}$) and lowest in the control ($1.62 \pm 0.02 \text{ mL}^{-1}$).

Clarias gariepinus fed D2 also recorded the highest ($P < 0.05$, ANCOVA) lysozyme activity values (2.00) than in fish fed D1 (1.93). In *C. gariepinus* fed D1, lysozyme activity decreased with an increase in handling times (Figure 3.8B). Fish handled for 45 minutes ($1.24 \pm 0.32 \text{ mL}^{-1}$) recorded the lowest lysozyme activity, and it was significantly different ($P < 0.05$) from control and fish handled for 15 minutes. In *C. gariepinus* fed D2, an increase in handling times also led to an increase in lysozyme activity (Figure 3.7B). However, there were no significant differences ($P > 0.05$) in lysozyme activity among handling times. The highest lysozyme activity was observed in fish handled for 45 minutes ($2.46 \pm 0.31 \text{ mL}^{-1}$) and lowest in control ($2.00 \pm 0.25 \text{ mL}^{-1}$) (Figure 3.8B).

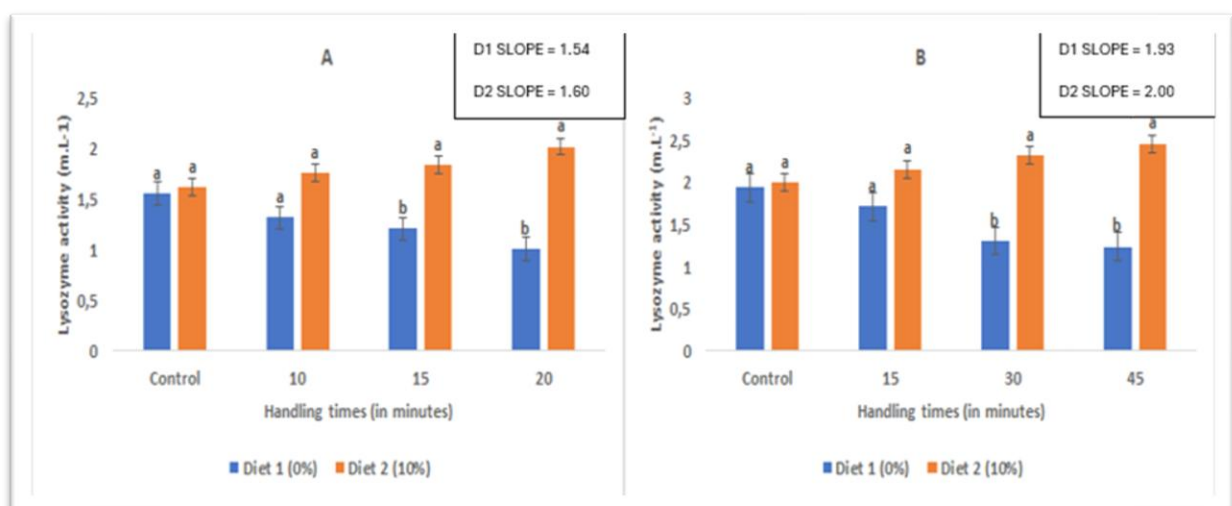


Figure 3. 8: The effect of different handling times on lysozyme activity of *O. mossambicus* (A) and *C. gariepinus* (B) fed with the 0% *A. afra*-based diet (D1) and

10% *A. afra*-based diet (D2). Bars with different letters are significantly different ($P < 0.05$, ANCOVA).

3.5.4.2 Plasma cortisol

Oreochromis mossambicus fed D2 recorded the lowest ($P < 0.05$, ANCOVA) plasma cortisol values (1.16) than in fish fed D1 (1.20). Plasma cortisol increased with handling times in *O. mossambicus* fed D1 (Figure 3.9A). Fish in control (1.26 ± 0.14 $\mu\text{g/ml}$) recorded the lowest plasma cortisol, and it was significantly different ($P < 0.05$) among all handling times. No significant difference ($P > 0.05$) in plasma cortisol was observed between fish handled for 10 minutes and 15 minutes. In *O. mossambicus* fed D2, an increase in handling times led to a decrease in plasma cortisol (Figure 3.9A). The lowest plasma cortisol was recorded in fish handled for 20 minutes (0.97 ± 0.21 $\mu\text{g/ml}$), and it was significantly different ($P < 0.05$) from control (2.61 ± 0.62 $\mu\text{g/ml}$), fish handled for 10 minutes (2.34 ± 0.02 $\mu\text{g/ml}$) and 15 minutes (2.21 ± 0.11 ng/ml). No significant difference was observed between control; fish handled for 10 and 15 minutes (Figure 3.9A).

An increase in handling times led to an increase in plasma cortisol in *C. gariepinus* fed D1 (Figure 3.9B). The lowest plasma cortisol was recorded in control (1.76 ± 0.00 $\mu\text{g/ml}$), and it was significantly different ($P < 0.05$) from fish handled for 15 minutes (2.96 ± 0.16 $\mu\text{g/ml}$), fish handled for 30 minutes (3.01 ± 0.39 $\mu\text{g/ml}$) and 45 minutes (3.22 ± 1.15 $\mu\text{g/ml}$). However, there were no significant differences ($P > 0.05$) in plasma cortisol among fish handled for 15, 30, and 45 minutes. In *C. gariepinus* fed D2, an increase in handling times also led to a decrease in plasma cortisol (Figure 3.9B). The lowest plasma cortisol was recorded in fish handled for 45 minutes (2.00 ± 0.37 $\mu\text{g/ml}$), and it was significantly different from control (2.46 ± 0.04 $\mu\text{g/ml}$), fish handled for 15 (2.32 ± 0.24 $\mu\text{g/ml}$) and 30 minutes (2.16 ± 0.47 $\mu\text{g/ml}$). A significant difference ($P < 0.05$) in plasma cortisol was observed between all the handling times (Figure 3.9B). *Clarias gariepinus* fed D1 recorded the highest ($P < 0.05$, ANCOVA) plasma cortisol values (1.06) than in fish fed D2 (1.18).

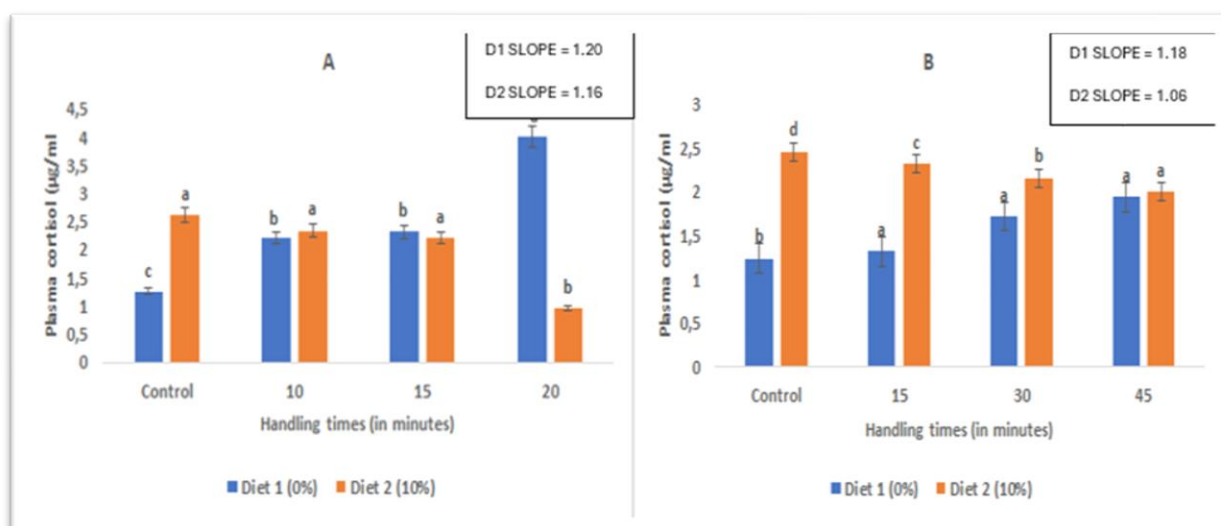


Figure 3. 9: The effect of different handling times on plasma cortisol of *O. mossambicus* (A) and *C. gariepinus* (B) fed with the 0% *A. afra*-based diet (D1) and 10% *A. afra*-based diet (D2). Bars with different letters differ significantly ($P < 0.05$).

All the results of this chapter are summarized in Table 3.4 and 3.5 for *O. mossambicus* and *C. gariepinus*, respectively.

Table 3. 4: Summary table of the results in *Oreochromis mossambicus*

Parameters	Relationship between D1 and D2
Opercular beat rate	Both D1 and D2 increased with handling times; however, D2 had a lower OBR.
Specific growth rate	Both D1 and D2 decreased with increasing handling times, although D2 had a higher SGR.
Feed conversion ratio	D1 and D2 increased with increased handling times; however, D2 had a lower FCR.
Feed intake	Both D1 and D2 decreased with an increase in handling times; however, D2 had a higher feed intake.
Survival rate	No mortality in D2, whereas the mortality rate increased with handling times in D1

WBC	Fish fed D1 decreased with increasing handling, and D2 increased with handling times.
RBC	As handling times increased, D2 had the greatest RBC values, although D1 and D2 declined as well.
HCT	Both D1 and D2 decreased with increasing handling times, although the highest HCT was observed in D2.
HGB	D1 decreased with increasing handling times, whereas D2 increased with handling times.
MCV	D1 decreased with increasing handling times. D2 also had the same trend with the highest values.
MCH	D1 decreased with an increase in handling time, and D2 increased with handling times.
MCHC	D1 decreased as handling times increased, while D2 increased in accordance.
Platelets	D1 decreased as handling times increased, while D2 increased in accordance.
Blood performance	D1 decreased with increasing handling times, whereas D2 increased with handling times.
Lysozyme activity	D1 decreased with increasing handling times, whereas D2 increased with handling times.
Plasma cortisol	D2 decreased with increasing handling times, whereas D1 increased with handling times.

Table 3. 5: Summary table of the results in *Clarias gariepinus*.

Parameters	Relationship between D1 and D2
Opercular beat rate	With increased handling times, D1 and D2 both increased; however, D2's OBR was lower.
Specific growth rate	D2 had a greater SGR than D1; however, both D1 and D2 declined as handling times increased.
Feed conversion ratio	Both D1 and D2 increased with an increase in handling times; however, D2 had a lower FCR.

Feed intake	While D2's feed intake was higher, both D1 and D2's declined with increased handling times.
Survival rate	No mortality in fish fed D2, whereas fish fed D1 decreased with an increase in handling times.
WBC	Fish fed D1 decreased with increasing handling, and D2 increased with handling times.
RBC	Both D1 and D2 decreased with increasing handling times; however, D2 had the highest RBC values.
HCT	Both D1 and D2 decreased with increasing handling times; however, D2 had the highest RBC values.
HGB	An increase in handling times led to a decrease in D1, while D2 increased with handling times.
MCV	An increase in handling times led to a decrease in D1, while D2 increased with handling times.
MCH	D1 decreased with increased handling times, whereas D2 had no discernible trend.
MCHC	D1 decreased with increased handling times, whereas D2 had no discernible trend.
Platelets	An increase in handling times led to a decrease in D1, while D2 increased with handling times.
Blood performance	D1 decreased with increasing handling times, whereas D2 increased with handling times.
Lysozyme activity	D1 decreased with increasing handling times, whereas D2 increased with handling times.
Plasma cortisol	An increase in handling times caused an increase in D1, whereas it caused a decrease in D2.

3.6 DISCUSSION

Opercular beat rate (OBR) significantly increased with handling times in both fish species fed with the 0% *A. afra*-based diet (D1) and 10% *A. afra*-based diet (D2). Opercular activity is a measure of oxygen consumption and metabolism in fish. Handling fish outside the water inevitably stresses them, and when they are put back in the water, oxygen consumption and metabolism increase. In this study, *O. mossambicus* and *C. gariepinus* fed with the 0% *A. afra*-based diet exhibited a higher opercular beat rate than those fed with the 10% *A. afra*-based diet. This is due to the effect of the 10% *A. afra*. An increase in opercular beat rate is associated with stress. This may indicate that fish fed with the 0% *A. afra*-based diet were more reactive to handling, leading to a higher opercular beat rate, which resulted in stress. In contrast, fish fed with the 10% *A. afra*-based diet might have had a different recovery rate, which resulted in a lower opercular beat rate compared to fish fed with the 0% *A. afra*-based diet. Although the opercular beat rate did not decrease with increasing handling times, this might be due to the effect of handling. Therefore, this may explain the potential effect of using the 10% *A. afra*-based diet, which helped to lessen stress by having a lower opercular beat rate. Previous studies also suggested that plants can be used to lower the opercular beat rate. Immanuel *et al.* (2009) and Oparaku *et al.* (2021) fed *C. gariepinus* juveniles with 0, 0.5, 1.0, 1.5, and 2.0% *Zingiber officinale* (ginger) meal for 6 weeks and found that fish fed 1.0% *Z. officinale* extracts had significantly lower opercular beat rate. This may also indicate that the 10% *A. afra*-based diet has properties that mitigate the response or alter both fish's physiological state.

The opercular beat rate can be influenced by several factors. Temperature and fish size are two key factors that will affect opercular beat rate (Samuel, 2020; McPhee *et al.*, 2023). In this study, the mean weight of *O. mossambicus* was 30.65 grams, and *C. gariepinus* was 38.36 grams. Smaller fish sizes are more active and have higher oxygen consumption, leading to higher opercular beat rates. Marnoto (2015) reported a higher opercular beat rate (233 OBR per minute) in smaller fish sizes of *O. mossambicus* (10.0 grams) handled for 4 minutes. In a similar study conducted by Gobi *et al.* (2018), handling *O. mossambicus* (42.14 grams) for 4 minutes did not affect its opercular beat rate. Temperature also plays a significant role in the response of fish to handling. Metabolic activity increases as temperature increases. The temperature

used in this study (28°C) is deemed to be the most appropriate temperature for both fish species. The significance of temperature in the capacity of fish to handle stress was illustrated by Oyelese (2007) in *C. gariepinus* cultured at a temperature of 34 °C and Sardella *et al.* (2004) in *O. mossambicus* cultured at a temperature of 32 °C who found that higher temperatures significantly increased the opercular beat rate of both fish species.

Both fish species responded differently to handling when kept at 28 °C temperature. For instance, when handled for 6 minutes, *C. gariepinus* opercular beat rate was significantly lower than in *O. mossambicus*. *Clarias gariepinus* are known for their ability to survive in low oxygen conditions due to their accessory respiratory organ called the suprabranchial arch (Hasin, 2022; Lisachov *et al.*, 2023). This adaptation may contribute to a lower opercular beat rate as it helps in breathing atmospheric air alongside oxygen from the water. Tilapia do not have a suprabranchial arch; they can utilize an air-water interface, which allows the exchange of oxygen and temperature regulation and assists in swimming up when handled (Mylnczenko, 2021). Whilst *C. gariepinus* has a suprabranchial arch that helps them when oxygen levels are low in the water, *O. mossambicus* on the other hand, can utilize an air-water interface when oxygen levels are low in the water. Since tilapia did not swim up to the air-water interface, it shows that the oxygen levels in the water were enough. The reason for tilapia not to swim up or engage in strenuous activities might be that it is trying to conserve energy while coping with stress.

Feed intake declined with an increase in handling times in both fish species fed with the 0% *A. afra*-based diet (D1) and 10% *A. afra*-based diet (D2). The decline in feed intake may be attributed to the stress in fish, thus affecting their appetite. Several studies have shown that fish appetite declines when fish are stressed by both intrinsic and extrinsic factors (Assan *et al.*, 2021). Extrinsic factors (handling, physical injury, and temperature) and intrinsic factors (neuroendocrine regulation, gut motility, immune function, and increased energy demands) may have affected the feed intake of both fish species. However, it is important to note that feed intake in both species was more reduced in fish fed with the 0% *A. afra*-based diet (D1) than in fish fed with the 10% *A. afra*-based diet (D2). This is consistent with the previous observation on the opercular beat rate. Foo and Lam (1993) also showed that *O. mossambicus* feed

intake declined when handled for 15 minutes. Adeyemo *et al.* (2009) indicated that handling *C. gariepinus* for 15 minutes can significantly affect feed intake.

The decline in feed intake is evident in specific growth rate and feed conversion ratio. The growth performance of both fish species fed with the 0% and 10% *A. afra*-based diets in terms of specific growth rate decreased with increasing handling times. The best SGR was observed in fish that were not handled. This could be attributed to increased oxygen demand, which disrupts feeding behavior and reduces appetite when fish are handled. Handling can induce behavioural changes in both *O. mossambicus* and *C. gariepinus*, such as reduced activity levels, increased hiding behavior, or altered social dynamics (Huntingford *et al.*, 2020). These behavioural adaptations may interfere with normal foraging activities and social interactions, further exacerbating the decline in growth performance (Ferreira *et al.*, 2022). These results agree with previous research on several fish species, *O. niloticus* (Shokr, 2012), *C. carpio* (Varga *et al.*, 2014), and red tilapia (Bernardino *et al.*, 2020). However, these fish species were not fed diets supplemented with ingredients containing antistress properties. Hoseini and Nodeh (2013) indicated that the supplementation of *Syzygium aromaticum* improved the growth performance and feed utilization in the diets of *Cyprinus carpio* (cyprinid). Furthermore, the authors reported that this plant is known to possess compounds with various functions, such as growth-promoting properties (Simtoe *et al.*, 2024). The results from the specific growth rate were consistent with the feed conversion ratio. Fish in control also recorded the best FCR compared to fish handled. This can be attributed to the same factors as indicated in the specific growth rate.

According to Aquaculture condition standards, FCR is expected to range between 1.2 – 1.8 for tilapia and 1.0 – 2.0 for catfish, and SGR between 2.0 – 3.5%/day and 2.5 – 4.0%/day respectively (El-Sayed, 2006; Faturoti and Akinbote, 1986; Tacon and Metian, 2008). However, in this study, the SGR (< 2.0 %/day) and feed intake values (< 2 g per fish/day) were quite low, wherein FCR (> 3) was quite high with accordance to the fish health standards. This might have been attributed to the effect of handling stress, which is known to elevate stress responses, disrupt metabolic efficiency, and reduce feeding performance (Barreto and Volpato, 2004). However, despite these

challenges, fish fed the *Artemisia afra*-supplemented diet exhibited notable resilience, maintaining measurable growth and stable opercular beat rates (OBR), and showing signs of enhanced immunity. This suggests that *Artemisia afra*, known for its bioactive and immunostimulatory properties, may have helped buffer the effects of stress by supporting immune function and mitigating physiological strain, consistent with findings from Reverter *et al.* (2014). Therefore, while performance metrics fell short of industry benchmarks, the results underscore the potential of *Artemisia afra* as a functional feed additive in improving fish health under stressful culture conditions.

The highest mortality rate was recorded in fish fed with the 0% *A. afra*-based diet. Stress from handling usually weakens fish and increases their risk of mortality (Sures and Nachev, 2022). No mortality was observed in fish fed with the 10% *A. afra*-based diet. Despite the fact that the 10% *A. afra*-based diet did not improve the growth performance and feed utilization of both fish species when handled, the response of both fish species to handling may indicate that the 10% *A. afra*-based diet reduced stress in the fish, since no mortalities were recorded. Ayanwale *et al.* (2020) reported that handling *C. gariepinus* for 30 minutes led to a higher mortality rate. In addition, Ali *et al.* (2020), in *O. niloticus*, also reported a higher mortality rate when handled for 15 minutes. This explains the positive effect of the 10% *A. afra*-based diet in increasing the survival rate of *O. mossambicus* and *C. gariepinus* when handled. Studies conducted by Alexander *et al.* (2010) indicated that *Tinospora cordifolia* increases the survival rate of *O. mossambicus* when subjected to different pH levels.

Plasma cortisol is a suitable indicator of stress in fish (Barton and Iwama, 1991). An increase in handling led to an increase in plasma cortisol in fish fed with the 0% *A. afra*-based diet. Increasing handling time has been reported to increase plasma cortisol and is associated with stress (Abdel-Tawwab, 2012). This may indicate that handling times stressed both *O. mossambicus* and *C. gariepinus*; hence, it resulted in elevated cortisol levels, which suppress the immune response, making fish more vulnerable to infections or diseases. Therefore, to cope with the current stressful event, immune system processes must be compromised to save energy. However, a reduced plasma cortisol level in fish fed with the 10% *A. afra*-based diet when subjected to stressful conditions may indicate the potential effect of *A. afra* to lessen

the response of the fish's endocrine system to stressors, which could have implications for the fish's ability to cope with future stressors. Similar observations have been recorded previously from handled fish fed with diets supplemented with medicinal plants in their feed additives. For instance, *Ocimum gratissimum* significantly reduced plasma cortisol levels in silver catfish bagre (*Rhamdia quelen*) when handled for 10 minutes (Corso *et al.*, 2019). *Ocimum gratissimum* contains active ingredients such as polyphenols, alkaloids, rosmarinic acid, flavonoids, quercetin, and rutin. It is known to possess compounds with various functions, such as growth-promoting and antioxidative properties (Abdel-Tawwab *et al.*, 2018). *Artemisia afra* contains active ingredients such as polyphenols, phenols, tannins, saponins, flavonoids, carotenoids, alkaloids, and Vitamin C, compounds with various functions such as antioxidant properties. This may explain why plasma cortisol was lower when fish were under stressful conditions.

Cazenave *et al.* (2005) reported that haematological parameters are not good indicators of stress in fish since they produce different results. In some cases, the increase in RBC, WBC HCT, HGB, MCV, MCH, MCHC, and platelets are used as an indicator of stress, and in some cases, they are not considered as stress indicators, because of the different results they produce. Therefore, it was important to introduce a new index, blood performance (BP). An increase in the blood performance of fish is associated with better health in fish (Esmaeili, 2021). The highest blood performance in *O. mossambicus* and *C. gariepinus* was recorded in control fed with the 0% *A. afra*-based diet. This may indicate that handling compromised both fish species' immune system. However, in fish fed with the 10% *A. afra*-based diet, blood performance increased with increasing handling times. This could suggest better health status of *O. mossambicus* and *C. gariepinus* where the fish were able to channel their energy to fight handling stress. The blood performance results indicate that *A. afra* may have led to better health responses in both *O. mossambicus* and *C. gariepinus* and provide a protection against handling stress. This shows that blood performance can measure fish stress levels accurately and provide helpful information.

An increase in lysozyme activity was also evident in fish fed with the 10% *A. afra*-based diet. However, handling times did not significantly impact lysozyme activity. In

O. mossambicus and *C. gariepinus*, the highest lysozyme activity was recorded in fish handled as opposed to fish not handled. In stressful conditions, elevated lysozyme activity may occur, as in innate immune responses. The increase in lysozyme activity indicates that there was high resistance against handling, thus strengthening the body's ability to fight against stressors and diseases. This could suggest that *A. afra* increased the activities of lysozyme in both species. This is consistent with the findings of Mbokane and Moyo (2018a, 2020a), which showed that *A. afra* concentration of 10% increased lysozyme activity in *O. mossambicus* and *C. gariepinus*. Additionally, this agrees with the results obtained by Hoseini *et al.* (2022) on *C. carpio* handled and fed an arginine-based diet. The authors showed that the incorporation of plants can improve lysozyme activity when both *O. mossambicus* and *C. gariepinus* are infected with pathogens. Lysozyme activity is essential for investigating fish's immune system (Ahmadifar *et al.*, 2020).

Conclusion

Handling stress affected the opercular beat rate and growth performance of both *O. mossambicus* and *C. gariepinus* fed with the 0% *A. afra*-based diet. However, handling stress did not affect the opercular beat rate and growth performance of both fish species fed with the 10% *A. afra*-based diet. Therefore, both fish species are physiologically better equipped to adapt to severe handling conditions when fed with the 10% *A. afra*-based diet. No mortalities were recorded in both fish fed with the 10% *A. afra*-based diet. The blood performance significantly increased with handling times. Plasma cortisol decreased with increasing handling times in both fish species fed with 10% *A. afra*. Lysozyme activity was not affected by handling times. Furthermore, the opercular beat rate, growth performance, and innate of *O. mossambicus* and *C. gariepinus* fed with the 10% *A. afra* were not affected by handling stress. In conclusion, the inclusion of a 10% *Artemisia afra*-based diet helped maintain the physiological response of both fish species during handling. This suggests that *A. afra* supplementation may have a protective or stress-mitigating effect, allowing the fish to better tolerate handling without adverse impacts on their overall health. Hence, the null hypothesis of this study is accepted.

CHAPTER 4

**EFFECTS OF FEED DEPRIVATION
PERIODS ON GROWTH
PERFORMANCE AND INNATE
IMMUNITY OF *OREOCHROMIS
MOSSAMBICUS* AND *CLARIAS
GARIEPINUS* FED *ARTEMISIA
AFRA*-BASED DIETS**

CHAPTER 4: EFFECTS OF FEED DEPRIVATION PERIODS ON GROWTH PERFORMANCE AND INNATE IMMUNITY OF *OREOCHROMIS MOSSAMBICUS* AND *CLARIAS GARIEPINUS* FED *ARTEMISIA AFRA*-BASED DIETS

4.1 INTRODUCTION

Feed deprivation is also one of the major stressors in aquaculture. Feed deprivation at fish farms is caused by poor fish husbandry practices, whereby fish farmers do not feed fish as regularly as they should. This occurs frequently in the small-scale aquaculture industries because of lack of resources and skills. Poor fish husbandry practices make it hard for fish farmers to realise economic gains. The effect of feed deprivation in the small-scale has rarely been evaluated within the context of rural aquaculture in Africa. However, feeding for alternate days or short periods of deprivation have been investigated, within the context of compensated feeding after deprivation. At some fish farms, they may deprive fish of feed for many days, and this may result in a decline in growth performance and increased stress levels (Hvas *et al.*, 2022). In addition, commercial feeds are expensive for small-scale fish farmers and most resources are channelled operational costs (Sahito *et al.*, 2022). Therefore, fish farmers ignore the recommended feeding regimes to reduce costs, thus compromising growth and production. Moreover, deprived fish are prone to stress and diseases.

Different fish species will respond differently to feed deprivation (Liu *et al.*, 2013). *Clarias gariepinus* is an opportunistic predator and *Oreochromis mossambicus* is an herbivorous fish. The response of these fish species to feed deprivation has been undertaken in a few studies (Davis and Gaylord, 2011; Abanikannda *et al.*, 2019). These species are widely cultured in Africa, mostly in rural areas with poor fish husbandry practices. It is, therefore, imperative to undertake feed deprivation studies on these two species. Some fish species showed poor growth rates and reduced innate immunity after feed deprivation. In herbivorous fish, Wang *et al.* (2005) showed that hybrid tilapia, *O. mossambicus* × *O. niloticus* starved for 4 days exhibited poor growth performance and reduced innate immunity. Additionally,

Sakyi *et al.* (2020) also observed poor growth and reduced innate immunity in *O. niloticus* starved for 7 days. In the predator fish, Offem *et al.* (2013) indicated that *Synodontis clarias* showed poor growth performance and innate immunity when starved for 15 days. Teryila *et al.* (2020) also showed poor growth performance and innate immunity in *H. longifilis* starved for 14 days. However, in *O. niloticus* and *C. anguillaris*, growth rates and innate immunity improved after feed deprivation, albeit for a short time (Zuo *et al.*, 2012; Song *et al.*, 2021). Hephher *et al.* (1983) indicated that *Tilapia sparmanii* starved for 5 days, had improved growth and immunity. Abdel-Hameid (2011) showed that *C. gariepinus* improved growth and enhanced innate immunity when starved for 7 days. These results show that both predatory and herbivorous fish are able to exhibit improved growth and innate immunity when starved for a few days as opposed to more extended periods.

The response of fish to feed deprivation depends not only on fish species but also on the size of the fish, temperature, and the production system (Ahmed *et al.*, 2020; Thorat *et al.*, 2023). Sakyi *et al.* (2020) reported that *O. mossambicus* weighing 20.2 grams was more negatively affected by feed deprivation than fish weighing 150.4 grams (Gabriel *et al.*, 2018). Higher temperatures can affect the response of fish to feed deprivation (Thorat *et al.*, 2023). Chen *et al.* (2022) indicated that *C. carpio* growth and innate immunity were more negatively affected by feed deprivation when cultured at a temperature of 34 °C than when cultured at a temperature of 28 °C.

The production system also affects the response of fish to feed deprivation (Mohanty *et al.*, 2021). Ebeling and Timmons (2012) showed that earthen ponds can negatively affect fish growth and innate immunity than recirculating aquaculture systems (RAS). Fish in earthen ponds cannot be starved because of zooplanktons and phytoplankton. However, different authors have come up with different deprivation tolerance times because these studies have been undertaken on different fish species, fish size, temperature, and production systems. This study will focus on sub-adult fish in *O. mossambicus* and *C. gariepinus*. The major limitations of most of these studies are that they focused on growth performance and ignored the effect of feed deprivation on

stress levels. This study will investigate the effect of feed deprivation on growth performance and stress levels in *O. mossambicus* and *C. gariepinus* after feeding with the 0% and 10% *A. afra*-based diet.

4.2. SPECIFIC OBJECTIVES

The specific objectives of this chapter were to:

1. Determine the effect of the *A. afra*-based diets on the opercular beat rate in *O. mossambicus* and *C. gariepinus* when subjected to different feed deprivation periods.
2. Determine the effect of the *A. afra*-based diets on growth performance and feed utilization in *O. mossambicus* and *C. gariepinus* when subjected to different feed deprivation periods.
3. Determine the effect of the *A. afra*-based diets on haematological and immunological parameters in *O. mossambicus* and *C. gariepinus* when subjected to different feed deprivation periods.

4.3 NULL HYPOTHESIS

The null hypothesis of this chapter was:

1. Feed deprivation does not affect the opercular beat rate of *O. mossambicus* and *C. gariepinus* fed with the *A. afra*-based diets.
2. Feed deprivation does not affect the growth performance and feed utilization of *O. mossambicus* and *C. gariepinus* fed with the *A. afra*-based diets.
3. Feed deprivation does not affect the growth performance and feed utilization of *O. mossambicus* and *C. gariepinus* fed with the *A. afra*-based diets.

4.4 MATERIALS AND METHODS

4.4.1 Fish acclimatization

Mozambique tilapia (360) [*O. mossambicus* (28.62 ± 3.15 g)] and the African sharptooth catfish (360) [*C. gariepinus* (35.42 ± 2.66 g)] were selected from fish bred and reared at the Aquaculture Research Unit, University of Limpopo. The experiment trials commenced on the 03rd July 2023 until 16th August 2023. A similar number of one hundred and eighty of each fish species, as used in the previous chapter, was randomly chosen from grow-out ponds and acclimatized to experimental conditions as described in chapter three under section 3.4.1.

4.4.2 Feed preparation

The *A. afra* procurement was explained in chapter three under section 3.4.2, respectively.

4.4.3 Proximate analysis

All the formulated diets were analysed for dry matter, crude protein, crude lipid, crude fibre, and gross energy following the guidelines stipulated by the Association of Official Analytical Chemists (AOAC International, 2012). The analyses for proximate analysis were carried out as described in chapter three under section 3.4.3.

4.4.4 Experimental system

The recirculating system, as shown in Figure 3.1, was used in this experiment as outlined in chapter three, under section 3.4.4.

4.4.5 Experimental design

The experimental systems used in this study were the same systems described in chapter three under section 3.4.5. *Oreochromis mossambicus* consisted of four feed deprivation periods per dietary treatment. Feed deprivation periods in *O. mossambicus* were control (0), 3, 6 and 9 days. Control denotes that there was no feed deprivation.

One fish was taken from the experimental tank and put in a separate glass tank (10 L) to assess opercular beat rate per minute twice daily (09:00hr and 15:00hr) (Monday, Wednesday, and Friday). Immediately after opercular beat rate inspection, fish were fed control (0% *A. afra*-based diet) and 10% *A. afra*-based diet for 3 days, until apparent satiation, twice daily (09:00hr and 15:00hr) for a duration of 45 days. In *C. gariepinus*, the same feed deprivation procedure was used, although the feed deprivation periods used were namely, control (0), 6, 12, and 18 days. Control denotes that there was no feed deprivation. Different feed deprivation periods were used because fish species used in this experiment are different and respond differently to feed deprivation.

After feeding with *A. afra*-based diets, fish were monitored daily for any clinical signs, abnormal behaviour, or mortalities from the starvation effect. Throughout the acclimatization period, environmental conditions were maintained as optimum as possible for *O. mossambicus* and *C. gariepinus*, as mentioned in chapter 3, under section 3.4.6.

4.4.6 Determination of growth performance indices

Feed intake, feed conversion ratio, specific growth rate and weight gain were determined as outlined in chapter 3, under section 3.4.7.

4.4.7 Haematological analyses

The haematological analyses such as WBC, RBC, HGB, HCT, MCV, MCH, MCHC, platelets, and glucose were determined as outlined in chapter 3, under section 3.4.8. Blood performance was calculated as outlined in chapter 3, section 3.4.8.

4.4.8 Immunological analyses

The lysozyme activity and plasma cortisol were determined as outlined in chapter 3, section 3.4.9.

4.4.9 Statistical analyses

Percentage data was arcsine transformed prior to carrying out regression analysis. The regressions were significant when $P < 0.05$. Analysis of covariance (ANCOVA) was used to test if there were significant differences between D1 (0% *A. afra*) and D2 (10% *A. afra*) regressions for opercular beat rate, specific growth rate, feed intake, and feed conversion ratio. The relationship between covariate (feed deprivation periods) and dependent variables (OBR, SGR, FCR, FI, and BP) was examined using a linear regression model ($y = aX + b$). Wherein “a” is the slope and “b” is the intercept. The slope represents the relationship between the covariate and dependent variables. Normality and homogeneity of variance was confirmed using the Shapiro-Wilk normality test and Levene test, respectively using R studio (version 4.3, 2023). Analysis of covariance (ANCOVA) was also used on haematological parameters (WBC, RBC, HGB, HCT, MCV, MCH, MCHC, and Platelets) and immunological parameters (lysozyme activity, and plasma cortisol) fed with the 0% and 10% *A. afra*-based diets.

4.5 RESULTS

4.5.1 Effect of feed deprivation periods on opercular beat rate

In *O. mossambicus* fed both D1 and D2, OBR was highest in fish deprived of feed for 9 days (Figure 4.1A). In D1 and D2, there was a positive linear relationship between OBR and feed deprivation periods. There were significant differences in D1 and D2 amongst feed deprivation periods. Opercular beat rate was higher ($P < 0.05$, ANVOVA) in fish fed D1 than in fish fed D2 (Figure 4.1A).

Similarly, feed deprivation periods showed a positive linear relationship with opercular beat rate in *C. gariepinus* fed with D1 and D2 (Figure 4.1B). The highest OBR was observed in fish deprived of feed for 18 days, and there were significant differences ($P < 0.05$, ANCOVA) among feed deprivation periods. Similar to *O. mossambicus*, fish fed D1 recorded a higher opercular beat rate than fish fed D2 (Figure 4.1B).

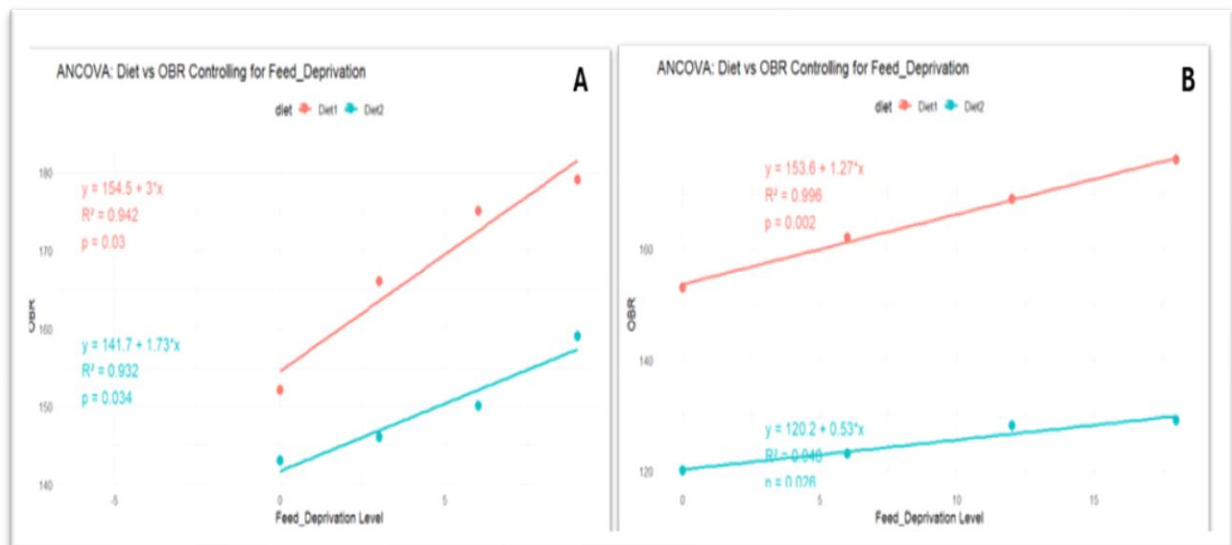


Figure 4. 1: Linear regression of feed deprivation periods on opercular beat rate of *O. mossambicus* (A) and *C. gariepinus* (B) fed D1 and D2.

4.5.2 Effect of feed deprivation on growth performance and feed utilization

4.5.2.1 Specific growth rate

There was a negative relationship ($P < 0.05$, ANCOVA) between SGR and feed deprivation periods in fish fed D1 ($R^2 = 0.999$) (Figure 4.2A). However, in fish fed D2, there was no significant relationship ($P > 0.05$, ANCOVA) between feed deprivation periods and SGR ($R^2 = 0.5322$). The highest SGR in fish fed D1 was recorded in control and the highest SGR in fish fed D2 was recorded in fish deprived of feed for 6 days. The SGR was higher in *O. mossambicus* fed D2 than in fish fed D1 (Figure 4.2A).

In *C. gariepinus* fed D1, a decrease in SGR was similarly observed with an increase in feed deprivation periods ($R^2 = 0.878$), and the highest ($P < 0.05$, ANCOVA) SGR was recorded in control (Figure 4.2B). *Clarias gariepinus* fed D2 also showed a similar trend as *O. mossambicus* fed D2, as there was no significant ($P > 0.05$, ANCOVA) relationship between SGR and feed deprivation periods (Figure 4.2B). The highest SGR was observed in fish deprived of feed for 12 days ($P > 0.05$, ANCOVA). Similar to *O. mossambicus*, SGR in *C. gariepinus* was higher in fish fed D2 (0.0588) than in fish fed D1 (0.0348) (Figure 4.2B).

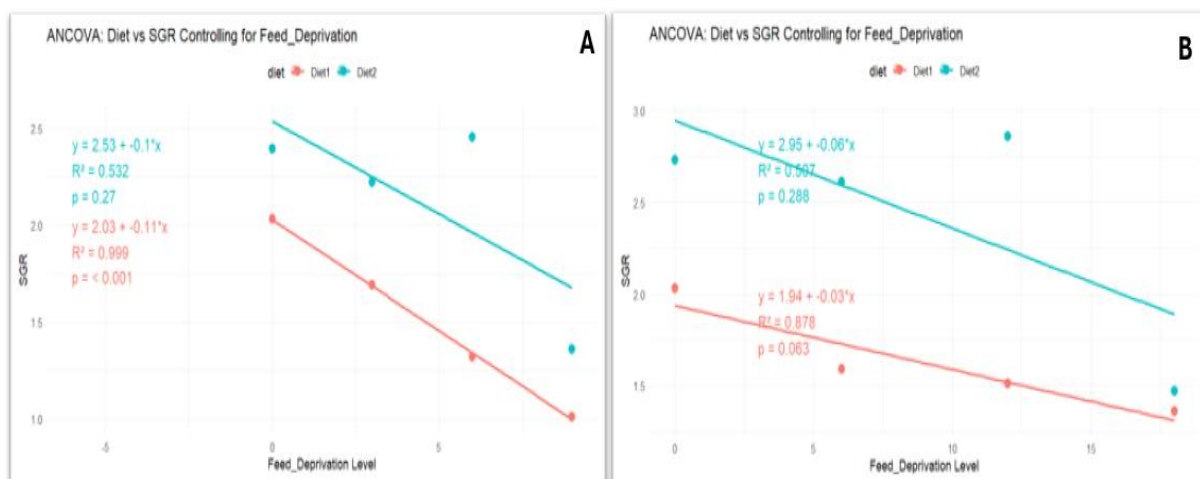


Figure 4. 2: Linear regression of feed deprivation periods on specific growth rate of *O. mossambicus* (A) and *C. gariepinus* (B) fed D1 and D2.

4.5.2.2 Feed conversion ratio

In *O. mossambicus* fed D1 and D2, FCR was highest in fish deprived of feed for 9 days (Figure 4.3A). Feed conversion ratio was higher in D1 than in fish fed D2. However, there were no significant differences ($P > 0.05$, ANCOVA) in fish fed D1 and D2 among feed deprivation periods. Both D1 and D2 showed positive linear relationships across feed deprivation periods.

Similarly, both D1 and D2 showed a positive linear relationship across feed deprivation periods (Figure 4.3B). The best FCR in D1 was observed in control, and the best FCR in D2 was observed in fish deprived of feed for 12 days. There were no significant differences ($P > 0.05$, ANCOVA) in fish fed D1 and D2 among feed deprivation periods. Feed conversion ratio was lower in *O. mossambicus* fed D2 than in fish fed D1 (Figure 4.3B).

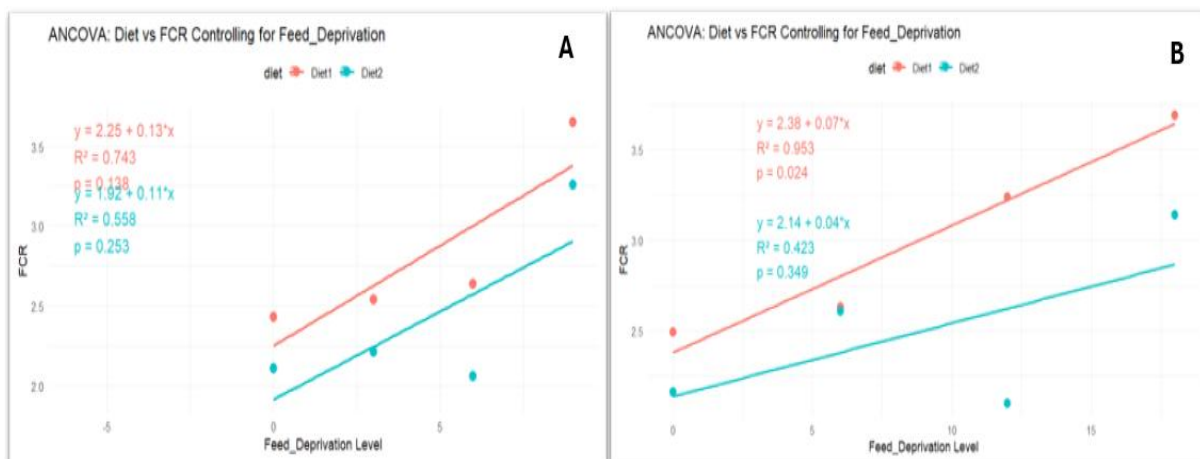


Figure 4. 3: Linear regression of feed deprivation periods on feed conversion ratio of *O. mossambicus* (A) and *C. gariepinus* (B) fed D1 and D2.

4.5.2.3 Feed intake

There was a positive linear relationship ($P < 0.05$, ANCOVA) between feed intake and feed deprivation periods in *O. mossambicus* fed D1 ($R^2 = 0.994$) (Figure 4.4A). In fish fed D2 ($R^2 = 0.326$), there was no significant relationship ($P > 0.05$, ANCOVA). The highest feed intake in fish fed D1 was recorded in control, wherein the D2 was highest

in fish deprived of feed for 6 days. Feed intake was much higher in fish fed D2 than in fish fed D1 (Figure 4.4A).

Clarias gariepinus fed D1 showed a significant decrease ($P < 0.05$, ANCOVA) in feed intake with an increase in feed deprivation periods ($R^2 = 0.727$) (Figure 4.4B). Fish fed D2 showed no significant relationship ($R^2 = 0.5431$) between feed intake and feed deprivation periods ($P > 0.05$, ANCOVA). The highest feed intake in fish fed D1 was recorded in control, wherein the D2 was highest in fish deprived of feed for 12 days. The feed intake was higher in *C. gariepinus* fed D2 than in fish fed D1 (Figure 4.4B).

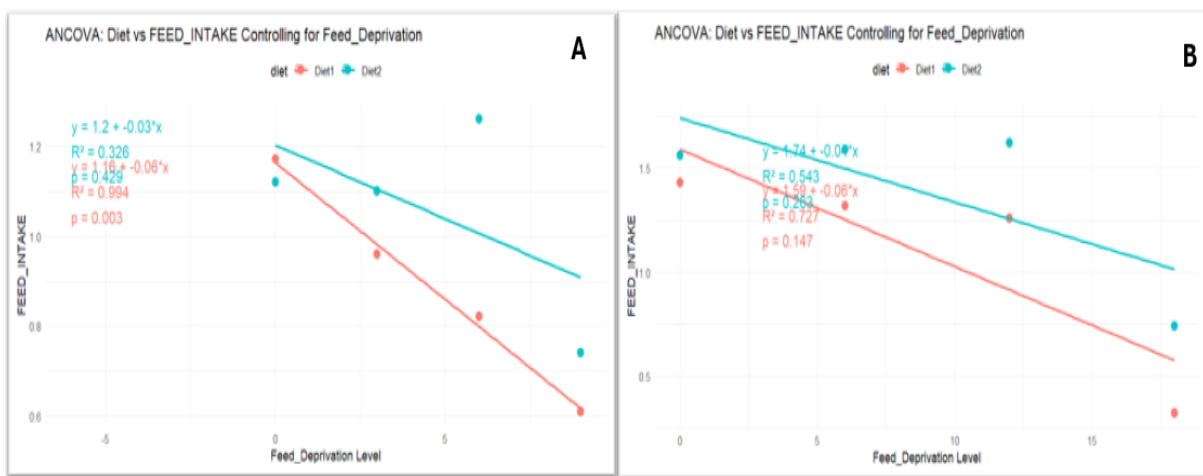


Figure 4. 4: Linear regression of feed deprivation periods on feed intake of *O. mossambicus* (A) and *C. gariepinus* (B) fed D1 and D2.

4.5.2.4 Survival rate

No mortality was observed in both fish species fed with the 10% *A. afra*-based diet (Figure 4.5A and 4.5B). The survival rate in both fish species fed with the 0% *A. afra*-based diet decreased significantly ($P < 0.05$) with increasing feed deprivation periods (Figure 4.5A and 4.5B). Both fish species fed D2 recorded the highest ($P < 0.05$, ANCOVA) slopes than in fish fed D1 (Figure 4.5A and 4.5B).

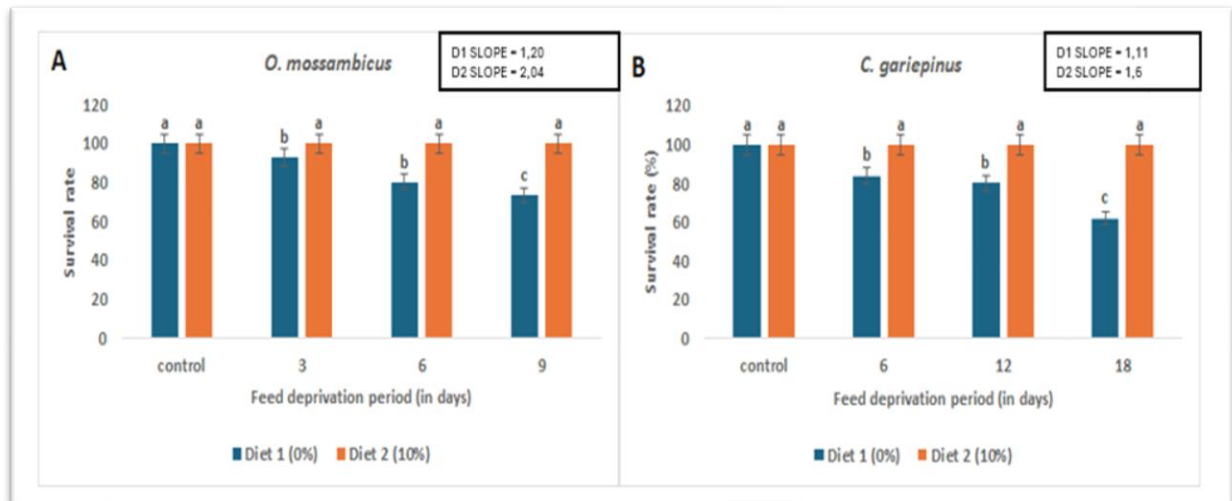


Figure 4. 5: The effect of different feed deprivation periods on survival rate (%) of *O. mossambicus* (A) and *C. gariepinus* (B) fed with the 0% *A. afra*-based diet (D1) and 10% *A. afra*-based diet (D2). Bars with different letters differ significantly ($P < 0.05$, ANCOVA).

4.5.3 Effect of feed deprivation on haematological parameters

In *O. mossambicus* fed D1, WBC, and RBC decreased with increasing feed deprivation periods (Table 4.1). The highest WBC and RBC were observed in control ($6.24 \pm 3.14 \times 10^6/L$) and ($10.41 \pm 2.63 \times 10^3/L$), respectively. Haematocrit cells and Haemoglobin also decreased with feed deprivation periods. The highest HCT and HCT were observed in control; however, there were no significant differences ($P > 0.05$) between the control and fish deprived of feed for 3 days. MCV, MCH, MCHC, and platelets followed the same trend as HGB and HCT (Table 4.1). Glucose levels significantly increased ($P < 0.05$) with feed deprivation periods. The lowest glucose level was recorded in control (5.36 ± 1.26 g/dL), and it was significantly different ($P < 0.05$) from other feed deprivation periods.

White blood cell counts in *O. mossambicus* fed D2 had no discernible pattern with feed deprivation periods (Table 4.1). The highest WBC count was recorded in fish deprived of feed for 6 days ($19.36 \pm 0.22 \times 10^6/L$). Red blood cells and Haematocrit cells decreased with increasing feed deprivation periods (Table 4.1). The highest ($P < 0.05$)

RBC and HCT counts were observed in control ($19.54 \pm 0.11 \times 10^3/L$) and ($3.91 \pm 2.15\%$), respectively. The MCV followed the same trend as the RBC (Table 4.1). The haemoglobin count had no discernible pattern with feed deprivation periods (Table 4.1). The highest HGB count was recorded in fish deprived of feed for 3 days, and there were no significant differences ($P > 0.05$) among feed deprivation periods. The MCH, MCHC, and platelets increased with feed deprivation periods. The highest ($P > 0.05$) values were observed in fish deprived of feed for 9 days. Glucose had no discernible pattern with feed deprivation periods (Table 4.1). The lowest glucose levels were observed in fish deprived of feed for 6 days (5.30 ± 0.36 g/dL), and it was significantly different ($P < 0.05$) from other feed deprivation periods (Table 4.1). Fish fed D2 had higher ($P < 0.05$, ANCOVA) WBC, RBC HCT, HGB, MCV, MCH, MCHC and platelets slopes than in fish fed D1.

White blood cells in *C. gariepinus* fed D1 decreased with increasing feed deprivation periods (Table 4.2). The highest WBC was observed in the control ($13.15 \pm 2.22 \times 10^6/L$), and it was significantly different ($P < 0.05$) from fish deprived of feed for 6, 12, and 18 days. However, no significant differences ($P > 0.05$) were observed among fish deprived of feed for 6, 12, and 18 days (Table 4.2). A similar trend was observed in HCT and HGB. The MCV declined with the feed deprivation period, and there were no significant differences ($P > 0.05$) among feed deprivation periods (Table 4.2). The MCH, MCHC, and platelets also followed a similar pattern. Glucose increased with feed deprivation periods (Table 4.2). The lowest glucose levels were observed in control (5.25 ± 1.25 g/dL), which was significantly different ($P < 0.05$) from fish deprived of feed for 12 and 18 days. No significant difference ($P > 0.05$) in glucose was observed between the control and fish deprived of feed for 3 days (Table 4.2).

In *C. gariepinus* fed D2, WBC counts also had no discernible pattern with feed deprivation (Table 4.2). The highest WBC count was recorded in fish deprived of feed for 12 days ($21.26 \pm 1.26 \times 10^6/L$), and it was significantly different ($P < 0.05$) from all feed deprivation periods. The RBC decreased with increasing feed deprivation periods (Table 4.2). The highest RBC count was recorded in control ($22.38 \pm 0.22 \times 10^3/L$), and it was significantly higher ($P < 0.05$) than in fish deprived of feed for 6, 12, and 18

days. The HCT followed the same trend as RBC (Table 4.2). Haemoglobin counts had no noticeable pattern with feed deprivation periods (Table 4.2). However, the highest HGB was observed in control (18.11 ± 2.26 g/dL), and it was significantly different ($P < 0.05$) from all feed deprivation periods. The MCV decreased with increasing feed deprivation periods. No significant differences ($P > 0.05$) were observed among feed deprivation treatments. The MCH increased with feed deprivation periods (Table 4.2). No significant differences ($P > 0.05$) were observed in MCH among feed deprivation periods. The MCHC had no discernible pattern with feed deprivation periods (Table 4.2). The highest ($P > 0.05$) MCHC was recorded in fish deprived of feed for 6 days. The highest platelet counts were recorded in fish deprived of feed for 18 days ($51.11 \pm 3.14 \times 10^6/L$), and it was significantly different ($P < 0.05$) from control and fish deprived of feed for 6 days. Glucose had no discernible pattern with feed deprivation periods (Table 4.2). The lowest glucose levels were observed in fish deprived of feed for 12 days (10.05 ± 1.58 g/dL), and it was significantly different ($P < 0.05$) from control, fish deprived of feed for 6 and 18 days (Table 4.2). Similarly, fish fed D2 had higher ($P < 0.05$, ANCOVA) WBC, RBC HCT, HGB, MCV, MCH, MCHC and platelets slopes than in fish fed D1.

Table 4. 1: Effect of feed deprivation periods on the haematological parameters of *O. mossambicus* fed with the 0% *A. afra*-based diet (D1) and 10% *A. afra*-based diet (D2) after 45 days of feeding (n=9). Values are the means of three replications \pm SD.

Haematological parameters	Feed deprivation periods							
	D1 (0%)				D2 (10%)			
	Control	3 days	6 days	9 days	Control	3 days	6 days	9 days
WBCs ($10^3/L$)	6.24 \pm 3.14 ^c	6.01 \pm 1.25 ^b	4.21 \pm 0.21 ^b	2.14 \pm 0.26 ^a	7.26 \pm 0.36 ^a	7.28 \pm 0.48 ^a	19.36 \pm 0.22 ^b	9.33 \pm 0.26 ^a
RBCs ($10^6/L$)	10.41 \pm 2.63 ^c	9.14 \pm 2.13 ^b	7.65 \pm 3.15 ^b	5.26 \pm 0.72 ^a	19.54 \pm 0.11 ^a	19.34 \pm 0.01 ^a	14.69 \pm 0.14 ^b	13.90 \pm 0.21 ^b
HCT (%)	4.69 \pm 0.26 ^b	3.62 \pm 0.11 ^b	2.24 \pm 0.26 ^a	1.26 \pm 0.35 ^a	3.91 \pm 2.15 ^a	2.18 \pm 2.34 ^b	2.01 \pm 2.22 ^b	1.05 \pm 3.14 ^c
HGB (g/dL)	16.26 \pm 2.14 ^a	13.15 \pm 3.65 ^a	11.53 \pm 1.11 ^b	10.24 \pm 1.26 ^b	14.39 \pm 1.17 ^a	16.32 \pm 1.19 ^a	12.30 \pm 1.24 ^a	10.11 \pm 1.34 ^a
MCV (fL)	206.22 \pm 5.36 ^c	115.20 \pm 5.11 ^b	75.53 \pm 6.78 ^a	55.26 \pm 8.11 ^a	216.22 \pm 5.36 ^a	215.20 \pm 5.11 ^a	79.53 \pm 6.78 ^b	75.26 \pm 8.11 ^b
MCH (pg)	60.15 \pm 1.26 ^a	52.23 \pm 3.45 ^b	49.32 \pm 1.25 ^b	43.19 \pm 3.52 ^b	54.18 \pm 3.14 ^a	55.33 \pm 2.48 ^a	55.36 \pm 2.47 ^a	60.17 \pm 2.59 ^a
MCHC (g/dL)	61.21 \pm 2.14 ^a	53.13 \pm 2.68 ^b	50.52 \pm 7.45 ^b	50.35 \pm 1.65 ^b	22.48 \pm 2.79 ^a	23.14 \pm 2.02 ^a	23.26 \pm 2.17 ^a	23.56 \pm 2.66 ^a
Platelets ($10^6/L$)	70.85 \pm 1.25 ^c	66.36 \pm 2.14 ^b	53.12 \pm 6.21 ^a	51.26 \pm 2.45 ^a	41.79 \pm 1.36 ^a	44.26 \pm 2.36 ^a	47.59 \pm 1.95 ^a	49.26 \pm 1.26 ^a
Glucose (g/dL)	5.36 \pm 1.26 ^c	7.24 \pm 1.59 ^b	7.59 \pm 3.45 ^b	9.55 \pm 2.42 ^a	8.36 \pm 1.23 ^a	9.25 \pm 1.11 ^a	5.30 \pm 0.36 ^b	8.42 \pm 1.14 ^a

In each row, different letters indicate significant differences between groups ($P < 0.05$) RBC: red blood cell counts; HCT: haematocrit; HGB: haemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; WBC: white blood cell counts. Values are presented as means \pm standard deviation.

Table 4. 2: Effect of feed deprivation periods on the haematological parameters of *C. gariepinus* fed with the 0% *A. afra*-based diet (D1) and 10% *A. afra*-based diet (D2) after 45 days of feeding (n=9). Values are the means of three replications \pm SD.

Haematological parameters	Feed deprivation periods							
	D1 (0%)				D2 (10%)			
	Control	6 days	12 days	18 days	Control	6 days	12 days	18 days
WBCs ($10^3/L$)	13.15 \pm 2.22 ^a	11.36 \pm 1.59 ^b	10.48 \pm 3.41 ^b	10.06 \pm 1.53 ^b	8.26 \pm 1.22 ^b	10.69 \pm 0.34 ^b	21.26 \pm 1.26 ^a	10.51 \pm 0.56 ^b
RBCs ($10^6/L$)	16.44 \pm 1.48 ^a	12.55 \pm 5.48 ^a	6.26 \pm 4.59 ^b	6.00 \pm 2.21 ^b	22.38 \pm 0.22 ^a	12.21 \pm 0.21 ^b	12.01 \pm 0.14 ^b	11.11 \pm 0.23 ^b
HCT (%)	23.36 \pm 4.28 ^a	20.11 \pm 4.21 ^a	19.45 \pm 3.21 ^b	15.22 \pm 1.49 ^b	28.14 \pm 3.14 ^a	17.22 \pm 1.32 ^b	16.61 \pm 4.33 ^b	10.16 \pm 3.14 ^b
HGB (g/dL)	6.22 \pm 1.65 ^a	6.02 \pm 1.36 ^a	3.15 \pm 0.24 ^b	2.68 \pm 0.13 ^b	18.11 \pm 2.26 ^a	14.23 \pm 2.14 ^b	17.35 \pm 3.36 ^b	12.32 \pm 1.29 ^b
MCV (fL)	96.21 \pm 2.45 ^a	92.24 \pm 3.66 ^a	90.00 \pm 6.25 ^a	86.36 \pm 0.36 ^a	176.25 \pm 1.36 ^a	176.22 \pm 2.14 ^a	176.13 \pm 6.26 ^a	169.62 \pm 5.23 ^a
MCH (pg)	70.25 \pm 2.14 ^a	64.36 \pm 2.55 ^a	63.21 \pm 5.24 ^a	60.66 \pm 3.69 ^a	44.76 \pm 3.14 ^a	44.79 \pm 2.33 ^a	45.33 \pm 3.22 ^a	45.89 \pm 2.14 ^a
MCHC (g/dL)	42.25 \pm 2.69 ^a	42.11 \pm 3.63 ^a	40.33 \pm 6.41 ^a	40.00 \pm 3.25 ^a	22.41 \pm 1.26 ^a	23.46 \pm 2.26 ^a	20.49 \pm 2.03 ^a	22.48 \pm 3.47 ^a
Platelets ($10^6/L$)	58.45 \pm 3.48 ^a	55.36 \pm 4.55 ^a	42.14 \pm 2.01 ^b	41.98 \pm 0.26 ^b	42.96 \pm 4.26 ^b	47.11 \pm 4.29 ^b	50.31 \pm 4.22 ^a	51.11 \pm 3.14 ^a
Glucose (g/dL)	5.25 \pm 1.25 ^b	10.36 \pm 2.14 ^a	12.69 \pm 2.31 ^a	16.65 \pm 1.29 ^a	13.25 \pm 1.15 ^a	12.41 \pm 1.36 ^a	10.05 \pm 1.58 ^b	12.26 \pm 1.26 ^a

In each row, different letters indicate significant differences between groups ($P < 0.05$) RBC: red blood cell counts; HCT: haematocrit; HGB: haemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; WBC: white blood cell counts. Values are presented as means \pm standard deviation.

4.5.3.1 Effect of feed deprivation on blood performance

Blood performance in *O. mossambicus* fed D1 decreased with an increase in feed deprivation periods ($R^2 = 0.899$) (Figure 4.6A). The highest blood performance in *O. mossambicus* fed D1 was recorded in control. Contrary to fish fed D1, blood performance in fish fed D2 increased with an increase in feed deprivation periods ($R^2 = 0.958$) (Figure 4.6A). However, the highest blood performance was recorded in fish deprived of feed for 9 days. No significant differences ($P > 0.05$, ANCOVA) were observed across feed deprivation periods in fish fed D1. Blood performance was much higher in fish fed D2 than in fish fed D1 (Figure 4.6A).

Clarias gariepinus blood performance showed a similar trend to *O. mossambicus* fed D1 and D2 (Figure 4.6B). However, fish fed D1 and D2 showed significant differences ($P < 0.05$) across feed deprivation treatments. Blood performance was higher in fish fed D2 than in fish fed D1 across the feed deprivation periods (Figure 4.6B).

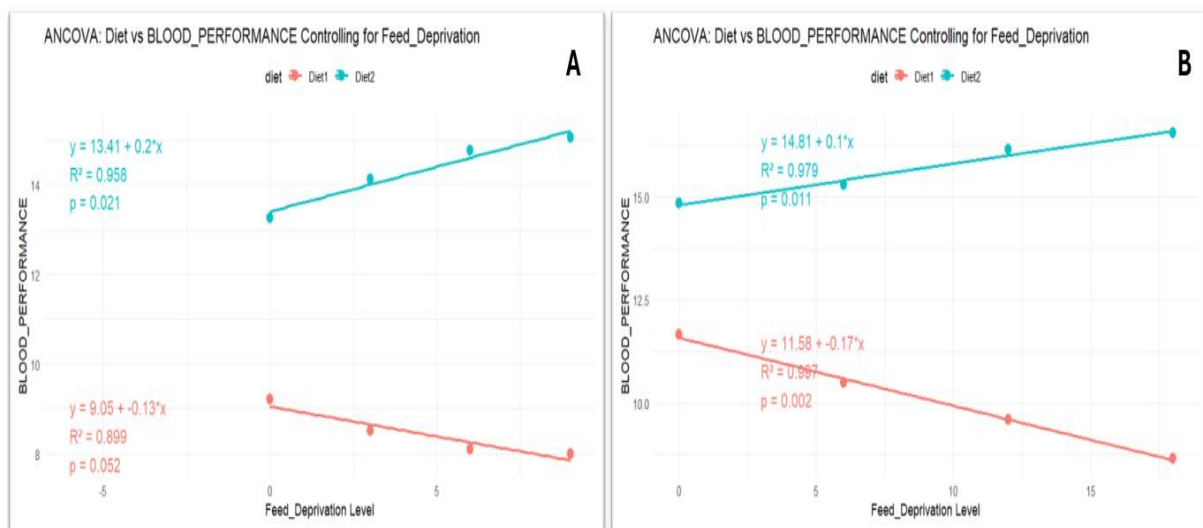


Figure 4. 6: Linear regression of feed deprivation periods on blood performance of *O. mossambicus* (A) and *C. gariepinus* (B) fed D1 and D2.

4.5.4. Effect of feed deprivation on immunological parameters

4.5.4.1 Lysozyme activity

Oreochromis mossambicus fed D1 recorded lowest ($P < 0.05$, ANCOVA) lysozyme activity values compared to D2 (Figure 4.7A). Lysozyme activity in *O. mossambicus* fed D1 declined with increasing feed deprivation periods (Figure 4.7A). The highest lysozyme activity was observed in the control ($15.15 \pm 5.23 \text{ mL}^{-1}$), which was significantly different ($P < 0.05$) from other feed deprivation periods. However, no significant differences ($P > 0.05$) in lysozyme activity were observed between control and fish deprived of food for 3 days. In *O. mossambicus* fed D2, lysozyme activity had no discernible pattern with feed deprivation periods (Figure 4.7A). The highest lysozyme activity was recorded in fish deprived of feed for 6 days ($22.16 \pm 3.47 \text{ mL}^{-1}$) and declined in fish deprived of feed for 9 days ($19.06 \pm 2.54 \text{ mL}^{-1}$) (Figure 4.7A). No significant variations ($P > 0.05$) in lysozyme activities were observed among feed deprivation periods.

Lysozyme activity in *C. gariepinus* fed D1 declined with increasing feed deprivation periods (Figure 4.7B). The highest lysozyme activity was observed in the control ($23.34 \pm 5.23 \text{ mL}^{-1}$), which was significantly different ($P < 0.05$) from other feed deprivation periods. However, no significant differences ($P > 0.05$) in lysozyme activity were observed between control and fish deprived of food for 6 days. In *C. gariepinus* fed D2, lysozyme activity also had no discernible pattern with feed deprivation periods, and there were no significant differences ($P > 0.05$) across feed deprivation periods (Figure 4.7B). The highest lysozyme activity was observed in fish deprived of feed for 12 days ($26.76 \pm 2.02 \text{ mL}^{-1}$). *Clarias gariepinus* lysozyme activity values also followed the same trend as *O. mossambicus* ($P < 0.05$, ANCOVA) (Figure 4.7B).

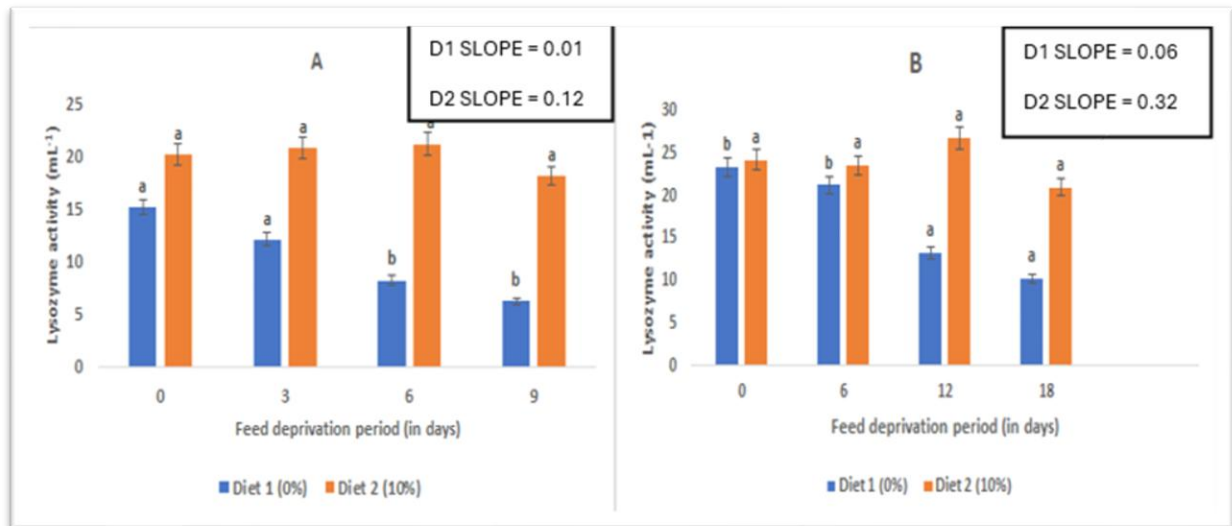


Figure 4. 7: The effect of different feed deprivation periods on Lysozyme activity of *O. mossambicus* (A) and *C. gariepinus* (B) fed with the 0% *A. afra*-based diet (D1) and 10% *A. afra*-based diet (D2). Different letters mean significant differences between feed deprivation treatments ($P < 0.05$).

4.5.4.2 Plasma cortisol

Plasma cortisol in *O. mossambicus* fed D1 increased with feed deprivation periods (Figure 4.8A). Fish in control recorded the lowest plasma cortisol ($5.02 \pm 1.28 \mu\text{g/ml}$), and it was significantly different ($P < 0.05$) from other feed deprivation periods. However, no significant difference ($P > 0.05$) was observed between fish deprived of food for 3, 6, and 9 days. In *O. mossambicus* fed D2, plasma cortisol had no discernible pattern with feed deprivation periods (Figure 4.8A). Fish deprived of feed for 6 days recorded the lowest plasma cortisol ($4.62 \pm 0.26 \mu\text{g/ml}$). However, no significant differences ($P > 0.05$) in plasma cortisol were observed among feed deprivation periods (Figure 4.8A). Plasma cortisol was much higher ($P < 0.05$, ANCOVA) in *O. mossambicus* fed D1 than in fish fed D2.

Plasma cortisol was also much higher ($P < 0.05$, ANCOVA) in *C. gariepinus* fed D1 than in fish fed D2. Plasma cortisol increased with feed deprivation periods in fish fed D1 (Figure 4.8B). The lowest plasma cortisol was recorded in control ($4.25 \pm 0.52 \mu\text{g/ml}$), which was significantly different ($P < 0.05$) from other feed deprivation periods.

However, no significant differences ($P > 0.05$) in plasma cortisol were observed between control and fish deprived of food for 6 days. Plasma cortisol in *C. gariepinus* fed D2 decreased with increasing feed deprivation periods (Figure 4.8B). The lowest plasma cortisol was recorded in fish deprived of feed for 12 days ($6.04 \pm 2.11 \mu\text{g/ml}$). However, no significant differences ($P > 0.05$) in plasma cortisol were observed among feed deprivation periods (Figure 4.8B).

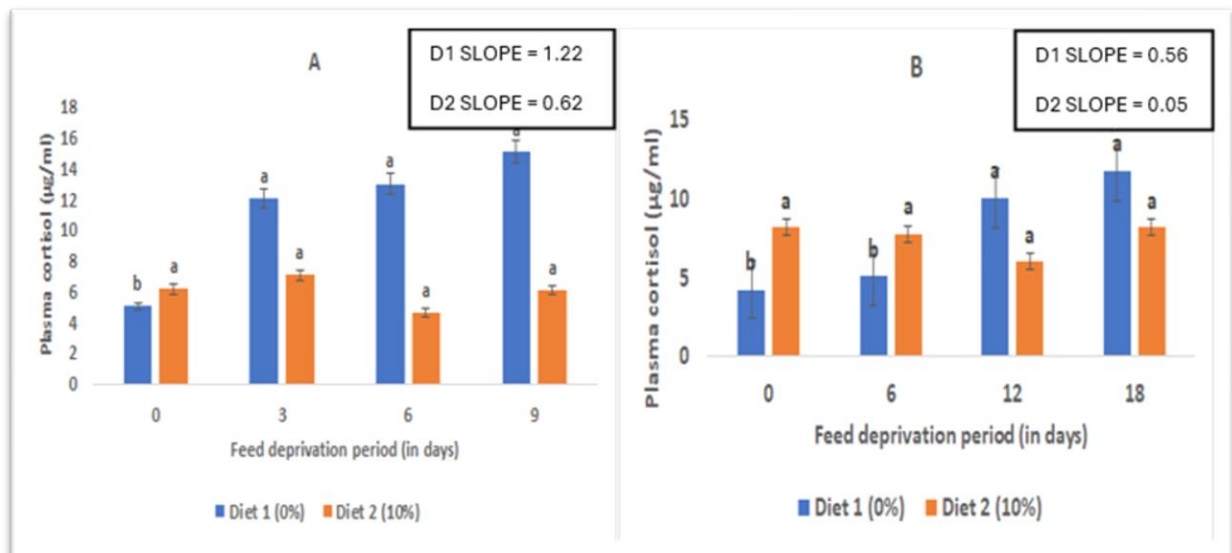


Figure 4. 8: The effect of different feed deprivation periods on Plasma cortisol of *O. mossambicus* (A) and *C. gariepinus* (B) fed with the 0% *A. afra*-based diet (D1) and 10% *A. afra*-based diet (D2). Different letters mean significant differences between starvation treatments ($P < 0.05$).

All the results of this chapter are summarized in Table 4.3 and 4.4 for *O. mossambicus* and *C. gariepinus*, respectively.

Table 4. 3: Summary table of the results in *Oreochromis mossambicus*

Parameters	Relationship between D1 and D2
Opercular beat rate	Both D1 and D2 increased with feed deprivation periods; however, D2 had a lower OBR.
Specific growth rate	Fish fed D1 decreased with increasing feed deprivation periods; however, there was no discernible trend with feed deprivation in D2, and the highest SGR was observed in fish deprived of feed for 6 days.
Feed conversion ratio	Fish fed D1 increased with an increase in feed deprivation periods; however, no noticeable pattern was observed between FCR and feed deprivation periods in D2, and the best FCR was observed in fish deprived of feed for 6 days.
Feed intake	While there was no discernible pattern with feed deprivation in D2, the highest feed intake was noted in fish that were deprived of feed for 6 days. D1 decreased as the period of feed deprivation increased.
Survival rate	The mortality rate in D1 increased with an increase in feed deprivation periods, while D2 had no mortality.
WBC	Fish fed D1 decreased with increasing feed deprivation periods; however, there was no discernible trend with feed deprivation in D2, and the highest WBC was observed in fish deprived of feed for 6 days.
RBC	Both D1 and D2 decreased with increasing feed deprivation periods, although the highest RBC was observed in D2.
HCT	With longer feed deprivation times, both D1 and D2 declined, while D2 showed the highest HCT.
HGB	While there was no discernible pattern with feed deprivation in D2, the highest HGB was observed in fish that were deprived of feed for 3 days. Feed intake in D1 decreased as the period of feed deprivation increased.

MCV	With longer feed deprivation times, both D1 and D2 declined, while D2 showed the highest MCV.
MCH	An increase in feed deprivation periods led to a decrease in D1, while D2 increased with feed deprivation periods.
MCHC	An increase in feed deprivation periods led to a decrease in D1, while D2 increased with feed deprivation periods.
Platelets	Fish fed D1 decreased with increasing feed deprivation periods, and D2 increased with feed deprivation periods.
Glucose	Fish fed D1 increased with an increase in feed deprivation periods; however, no noticeable pattern was observed between glucose and feed deprivation periods in D2, and the best glucose level was observed in fish deprived of feed for 6 days.
Blood performance	Fish fed D1 decreased with increasing feed deprivation periods, and D2 increased with feed deprivation periods.
Lysozyme activity	D1 decreased as feed deprivation periods increased, while D2 increased in accordance.
Plasma cortisol	While there was no discernible pattern with feed deprivation in D2, the lowest plasma cortisol was noted in fish that were deprived of feed for 3 days. D1 decreased as the period of feed deprivation increased.

Table 4. 4: Summary table of results in *Clarias gariepinus*

Parameters	Relationship between D1 and D2
Opercular beat rate	D1 and D2 increased with increased feed deprivation periods; however, D2 had a lower OBR.
Specific growth rate	While there was no discernible pattern with feed deprivation in D2, the highest SGR was noted in fish that were deprived of feed for 12 days. D1 decreased as the period of feed deprivation increased.
Feed conversion ratio	Fish fed D1 increased with an increase in feed deprivation periods; however, no noticeable pattern was observed between FCR and

	feed deprivation periods in fish fed D2, and the best FCR was observed in fish deprived of feed for 12 days.
Feed intake	Fish fed D1 decreased with increasing feed deprivation periods; however, there was no discernible trend with feed deprivation in D2, and the highest SGR was observed in fish deprived of feed for 12 days.
Survival rate	No mortality in D2, whereas the mortality rate increased with feed deprivation periods in D1.
WBC	Fish fed D1 decreased with increasing feed deprivation periods; however, there was no discernible trend with feed deprivation in D2, and the highest WBC was observed in fish deprived of feed for 12 days.
RBC	Both D1 and D2 decreased with an increase in feed deprivation periods; however, D2 had a higher RBC.
HCT	Both D1 and D2 decreased with increasing feed deprivation periods, although the highest HCT was observed in D2.
HGB	Fish fed D1 decreased with increasing feed deprivation periods; however, there was no discernible trend with feed deprivation in D2, and the highest WBC was observed in the control group.
MCV	D1 and D2 increased with increased feed deprivation periods; however, D2 had higher MCV values.
MCH	D1 decreased with increasing feed deprivation periods, whereas D2 increased with feed deprivation periods.
MCHC	While there was no discernible pattern with feed deprivation in D2, the highest MCH was noted in fish that were deprived of feed for 6 days. D1 decreased as the period of feed deprivation increased.
Platelets	Fish fed D1 decreased with increasing feed deprivation periods, and D2 increased with feed deprivation periods.
Glucose	Fish fed D1 increased with an increase in feed deprivation periods; however, no noticeable pattern was observed between glucose and feed deprivation periods in D2, and the best glucose level was observed in fish deprived of feed for 12 days.

Blood performance	D1 decreased as feed deprivation periods increased, while D2 increased in accordance.
Lysozyme activity	D1 decreased with increasing feed deprivation periods, whereas D2 increased with feed deprivation periods.
Plasma cortisol	While there was no discernible pattern with feed deprivation in D2, the lowest plasma cortisol was noted in fish that were deprived of feed for 6 days. D1 decreased as the period of feed deprivation increased.

4.6 DISCUSSION

The opercular beat rate in both fish species fed with the 0% and 10% *A. afra*-based diets increased with an increase in feed deprivation periods. However, fish fed with the 0% *A. afra*-based diet had a higher opercular beat rate than fish fed with the 10% *A. afra*-based diet. This may indicate that feed deprivation periods stressed the fish fed with the 0% *A. afra*-based diet, and fish were not able to adapt to feed deprivation periods when compared to 10% *A. afra*-based diet. Additionally, this may indicate that *A. afra* has anti-inflammatory properties, which may help reduce any respiratory inflammation or irritation in the fish gills, wherein a fish may exhibit a lower metabolic rate, leading to a lower opercular beat rate. This may have reduced stress levels in both *O. mossambicus* and *C. gariepinus*. Gouda and Ganesh (2023) indicated that when *O. mossambicus* was starved for 6 days, the fish was stressed. The authors reported that the opercular beat rate in fish deprived of feed was significantly higher than the fish fed on a daily basis. Mandefro *et al.* (2024) also reported an increase in the opercular beat rate of *O. niloticus* starved for 6 days. Allali *et al.* (2020) and Ugah (2021) also indicated that the feed deprivation period (12 days) affects the *C. gariepinus* opercular beat rate. However, fish fed with the 10% *A. afra*-based diet were not as stressed as fish fed with the 0% *A. afra*-based diet, and this shows that *A. afra* may have alleviated stress caused by feed deprivation, leading to more efficient respiration.

In both fish species fed with the 0% *A. afra*-based diet, feed intake declined with increasing feed deprivation periods. The amount of feed remaining in the stomach is one of the critical factors that affects appetite in fish (Jia *et al.*, 2021). Prolonged deprivation periods may have stressed the fish fed with the 0% *A. afra*-based diet; hence, feed intake declined. Depriving tilapia of feed for 24 hours would lead to a point whereby it is possible for them to eat more because there would be feed remaining in the stomach, and this would increase their appetite (Sakyi *et al.*, 2020). Several studies have shown that tilapia stomach would be empty after 3 days of feed deprivation (Chan *et al.*, 2008; El-Araby *et al.*, 2020). Since the stomach was empty, they did not eat more, which shows that they were somewhat stressed in fish fed with the 0% *A. afra*-based diet. Sakyi *et al.* (2021) also recorded a decline in feed intake in *O. niloticus* starved for 3 days. However, *O. mossambicus* and *C. gariepinus* fed with

the 10% *A. afra*-based diet had a higher feed intake when deprived of food for 6 days and 12 days, respectively. However, there was a decline in *O. mossambicus* deprived of feed for 9 days and *C. gariepinus* deprived of feed for 18 days. This may indicate that the 10% *A. afra*-based diet may have helped in maintaining gut health during the 6-day feed deprivation period in tilapia, which allowed better digestion and higher feed intake when the food was reintroduced. The case is similar to *C. gariepinus*, deprived of food for 12 days. Therefore, the decline in feed intake after 9 days (*O. mossambicus*) and 18 days (*C. gariepinus*) may have been due to prolonged starvation, which caused metabolic suppression and stress in fish. This suggests that the 10% *A. afra*-based diet is likely to enhance the feeding response after a short-term feed deprivation, but these benefits may not be enough to overcome the physiological constraints of prolonged feed deprivation of 9 days in tilapia and 18 days in catfish.

Specific growth rates in both *O. mossambicus* and *C. gariepinus* fed with the 0% *A. afra*-based diet also declined with increasing feed deprivation periods. This is explained by the reduced feed intake observed. This agrees with studies conducted by Abanikannda *et al.* (2019) in *C. gariepinus* and Sakyi *et al.* (2021) in *O. niloticus*, who showed that SGR declined with feed deprivation periods. The results from the SGR were consistent with FCR. Feed conversion increased in both fish species fed with the 0% *A. afra*-based diet. However, the incorporation of 10% *A. afra*-based diet-based in the diets of *O. mossambicus* and *C. gariepinus* led to a higher SGR when deprived of food for 6 days and 12 days, respectively. This can be explained by the same reason as in feed intake. Fish fed with the 0% *A. afra*-based diet had a higher mortality rate than fish fed with the 10% *A. afra*-based diet. No mortality was recorded in both fish species fed with the 10% *A. afra*-based diet. This probably shows the potential effect of using the 10% *A. afra*-based diet in the diets of tilapia and catfish. Abdel-Tawwab *et al.* (2006) and Ugah (2021) reported that depriving fish of feed can lead to higher mortality rates. The results showed that SGR (< 2.0 %/day) and feed intake values (< 2 g per fish/day) were quite low, wherein FCR (> 3) was quite high with accordance to the fish health standards. This is attributed to the same factors as mentioned in chapter 3.

Feed deprivation periods influenced plasma cortisol in both fish species fed with the 0% *A. afra*-based diet. At the biochemical level, plasma cortisol indicated that fish were stressed. These results are further confirmed by glucose levels. Glucose levels increased with increasing feed deprivation periods in both *O. mossambicus* and *C. gariepinus* fed with the 0% *A. afra*-based diet. However, at the 10% *A. afra*-based diet inclusion level, plasma cortisol and glucose levels indicated that both fish species were not stressed. This corresponds with the study conducted by Navarro *et al.* (2016), where plasma cortisol did not differ significantly across feed deprivation periods in *O. niloticus* fed *Syzygium aromaticum* (clove oil). *Syzygium aromaticum* is known to have active ingredients such as eugenol, polyphenols, thymol, carvacrol, cinnamaldehyde, rhamnetin, and oleanolic acid. Teles *et al.* (2019) reported that clove oil can also act as a potent antioxidant in fish culture since it reduces plasma cortisol levels in *Sparus aurata* (gilthead sea bream) under stressful conditions. *Artemisia afra* also contains active biological compounds such as polyphenols, phenols, tannins, saponins, flavonoids, carotenoids, alkaloids, and Vitamin C. This may explain the reason plasma cortisol levels did not rise when fish were under feed deprivation stress. Glucose levels also declined as feed deprivation periods increased. Low glucose levels indicate that fish species were not stressed (Jentoft *et al.*, 2005). This again may be explained by the positive effect of the 10% *A. afra*-based diet on the physiology of the fish, which helped to reduce stress.

As mentioned in chapter 3, haematological parameters (WBC, RBC, HCT, HGB, MCV, MCH, MCHC, and platelets) are not good indicators of stress in fish because they give contradictory results. Abdel-Moneim *et al.* (2020) indicated that the increase in WBC, RBC, HCT, and HGB shows that fish are not stressed. However, Shahjahan *et al.* (2022) argued that the increase in these parameters indicates that the fish is stressed. In this study, haematological parameters such as RBC, HGB, HCT, and MCV decreased with feed deprivation, whereas MCH, MCHC, and platelets increased. Therefore, it is difficult to conclude whether fish were stressed or not. Hence, this study used a new index called blood performance to evaluate if fish were stressed or not. An increase in the blood performance of fish is associated with better health in fish (Esmaeili, 2021). The highest blood performance was observed in both *O. mossambicus* and *C. gariepinus*, which were deprived of feed for 9 days and 18 days,

respectively, and fed with the 10% *A. afra*-based diet. This suggest better health status in both fish species, whereby they were able to channel their energy to fight feed deprivation stress when fed with *A. afra*-based diets. Fish fed with the 10% *A. afra*-based diet had the highest blood performance compared to fish fed with the 0% *A. afra*-based diet. This is consistent with the previous observations from the opercular beat rate and growth performance indices (feed intake, specific growth rate, and feed conversion ratio). This demonstrates that blood performance can give reliable results, and it can be used to monitor stress levels in fish. Additionally, these results probably indicate that the 10% *A. afra*-based diet may have stimulated better health responses in both fish species when starved for longer periods.

Lysozyme activity increased with feed deprivation periods in both *O. mossambicus* and *C. gariepinus* fed with the 10% *A. afra*-based diet. An increase in lysozyme activity is associated with increased immune response (Magouz *et al.*, 2021). In this study, fish fed with the 10% *A. afra*-based diet had the highest lysozyme activity compared to fish fed with the 0% *A. afra*-based diet. This may indicate that the 10% *A. afra*-based diet played a role in the immune response of the fish. Mbokane and Moyo (2018a; 2020a) indicated that an *A. afra* concentration of 10% elicits an optimum immune response in both *O. mossambicus* and *C. gariepinus*. The results obtained in this chapter are consistent with the findings obtained from the previous chapter. This shows that *A. afra* can be utilized in the diets of *O. mossambicus* and *C. gariepinus* to enhance immunity in stressed fish due to feed deprivation.

Conclusion

The best growth performance in *O. mossambicus* and *C. gariepinus* fed with the 10% *A. afra*-based diet in terms of SGR, FCR, and feed intake was observed in fish deprived of feed for 6 days. *Clarias gariepinus* showed the best growth performance in terms of SGR, FCR, and feed intake when deprived of feed for 12 days. However, feed deprivation periods affected both species' opercular beat rate, growth performance, and innate immunity when fed with 10% *A. afra*. Blood performance and lysozyme activity increased with increasing feed deprivation periods in fish fed with the 10% *A. afra*-based diet. *Oreochromis mossambicus* was deprived of feed for 6

days, and *C. gariepinus* was deprived of feed for 12 days and recorded the lowest plasma cortisol and glucose levels. Therefore, it is recommended to deprive *Oreochromis mossambicus* for 6 days and *Clarias gariepinus* for 12 days, followed by 3 days of refeeding with a 10% *Artemisia afra*-based diet, to optimize growth performance and innate immunity. The recommended maximum feed deprivation periods for *Oreochromis* spp. (3-7 days) and *Clarias gariepinus* (7-14 days). Therefore, this strategy is considered suitable provided that deprivation does not compromise fish welfare, as indicated by the maintenance of normal opercular rate, survival, behavior, and stress biomarkers during the trial period. In conclusion, the opercular beat rate, growth performance, and innate of *O. mossambicus* and *C. gariepinus* fed with the 10% *A. afra* were not affected by feed deprivation periods. This shows that both species fed the 10% *A. afra*-based diet can adapt to the feed deprivation periods compared to fish fed with the 0% *A. afra*-based diet. Thus, the null hypothesis is accepted.

CHAPTER 5

**EFFECTS OF STOCKING DENSITY
ON GROWTH PERFORMANCE AND
INNATE IMMUNITY OF
OREOCHROMIS MOSSAMBICUS
AND *CLARIAS GARIEPINUS* FED
ARTEMISIA AFRA-BASED DIETS**

CHAPTER 5. EFFECTS OF STOCKING DENSITY ON GROWTH PERFORMANCE AND INNATE IMMUNITY OF *OREOCHROMIS MOSSAMBICUS* AND *CLARIAS GARIEPINUS* FED *ARTEMISIA AFRA*-BASED DIETS

5.1 INTRODUCTION

In most fish species, high stocking density of 3.12 kg/m³ to 18 kg/m³ causes chronic stress, and this ultimately leads to the suppression of both innate and adaptive immune responses (Shourbela *et al.*, 2017; Adineh *et al.*, 2019). High stocking densities can lead to competition for resources, whereby fish compete for limited resources such as food, space, and oxygen. This can lead to increased aggression and social stress. Additionally, it can lead to poor water quality, which can stress fish physiologically and compromise their immune function. Sahoo *et al.* (2004) and Nwipie *et al.* (2015) observed the best growth and innate immunity of *C. gariepinus* at a low stocking density of 0.56 kg/m³ to 1.75 kg/m³, while Dasuki *et al.* (2013) observed the best growth and innate immunity in *C. gariepinus* at a medium stocking density of 1.89 kg/m³ to 2.95 kg/m³. However, in tilapia, low stocking density leads to growth regression, which is associated with stress. This may be because tilapia are social fish that establish hierarchies within their groups, and they are often more efficient feeders in higher-density environments due to the competitive nature of their feeding behaviour (Asase, 2013). At low-density, the reduced competition can lead to overfeeding by some individuals and underfeeding by others, causing inconsistent growth rates (Mohammady *et al.*, 2023). Additionally, lower densities might reduce the stimulus to feed actively, leading to reduced overall food intake. Al Jerian (1998) observed the best growth performance and innate immunity in *O. mossambicus* at a high stocking density of 4.5 kg/m³. Sabwa *et al.* (2020) and Al-Tawaha *et al.* (2021) observed the best growth performance and innate immunity in *O. niloticus*, at a medium stocking density of 2.50 kg/m³ and a high stocking density of 10 kg/m³, respectively. Therefore, the stocking densities used in these studies are consistent with densities used commercially in semi-intensive to moderately intensive systems, especially in pond and cage culture. However, they may not fully represent the higher densities used in intensive recirculating systems (RAS) or tank-based production, which can reach 30–

60+ kg/m³. Further studies at those levels would be valuable to broaden the commercial applicability.

Several studies have focused on the effect of stocking density on growth performance and stress levels in *O. mossambicus* (Wu *et al.*, 2018; Harahap *et al.*, 2023) and *C. gariepinus* (Mulugeta, 2022; Wenzel *et al.*, 2022). However, it is clear that there is limited information on reducing stress levels by using plant-based products to lower stress levels of *O. mossambicus* and *C. gariepinus* reared at different stocking densities. Supplementing diets with plant-based products can be helpful in reducing stress in fish culture (Acar *et al.*, 2015). More comparative studies are needed to address the required stocking density when fish are fed with diets that contain plant-based products. *Artemisia afra* has been shown to contain bioactive compounds such as polyphenols, phenols, tannins, saponins, flavonoids, alkaloids, carotenoids, and vitamin C, which can boost the immunity of fish. Therefore, the research question of this study is to explore how *A. afra* can improve growth and reduce stress in *O. mossambicus* and *C. gariepinus* in different stocking densities?

5.2 SPECIFIC OBJECTIVES

The specific objectives of this chapter were to:

1. Determine the effect of *A. afra*-based diets on the opercular beat rate in *O. mossambicus* and *C. gariepinus* reared at different stocking densities.
2. Determine the effect of *A. afra*-based diets on growth performance and feed utilization in *O. mossambicus* and *C. gariepinus* reared at different stocking densities.
3. Determine the effect of *A. afra*-based diets on haematological and immunological parameters in *O. mossambicus* and *C. gariepinus* reared at different stocking densities.

5.3 NULL HYPOTHESIS

The null hypotheses of this chapter were:

1. Different stocking densities do not affect the opercular beat rate of *O. mossambicus* and *C. gariepinus* fed with the *A. afra*-based diets.
2. Different stocking densities do not affect the growth performance and feed utilization of *O. mossambicus* and *C. gariepinus* fed with the *A. afra*-based diets.
3. Different stocking densities do not affect the haematological and immunological indices of *O. mossambicus* and *C. gariepinus* fed with the *A. afra*-based diets.

5.4 MATERIALS AND METHODS

5.4.1 Feed preparation

The *A. afra* procurement was explained in chapter three under section [3.4.2](#).

5.4.2 Proximate analysis

All the formulated diets were analysed for dry matter, crude protein, crude lipid, crude fibre, and gross energy, following the guidelines stipulated by the Association of Official Analytical Chemists (AOAC, 2012) International. The analyses for proximate analysis were carried out as described in chapter three under section [3.4.3](#).

5.4.3 Experimental location

The study was undertaken at the Aquaculture Research Unit, University of Limpopo, South Africa. The trials of this experiment commenced on the 21st of August 2023 until 11th September 2023.

5.4.4 Experimental system

The recirculating system, as shown in Figure 3.1, was used in this experiment as outlined in chapter three, under section [3.4.4](#).

5.4.5 Experimental design

Oreochromis mossambicus (480) with an average mean weight of 35.39 ± 3.41 g and *Clarias gariepinus* (480) with an average mean weight of 32.30 ± 4.26 g sub-adults were stocked in 36 recirculating aquaculture system (RAS) (18 tanks per species) using the experimental system described in chapter three under section 3.4.5. The research question of this experiment was to assess whether the inclusions of *A. afra* in the diets of *O. mossambicus* and *C. gariepinus* would reduce stress when stocked at different densities. The experiment was replicated three times. One fish was taken from the experimental tank and put in a separate glass tank (10 L) to assess the opercular beat rate per minute twice daily (09:00hr and 15:00hr) (Monday, Wednesday, and Friday). The fish were fed the 0% *A. afra*-based diet and 10% *A. afra*-based diets twice daily (09:00 hr and 15:00 hr) until apparent satiation. The experiment occurred over a period of 21 days. The stocking density experiment was limited to 21 days. This shorter duration was sufficient to observe the acute effects of elevated stocking density, such as immediate stress responses, increased competition for resources, and rapid changes in growth performance or opercular beat rate. Both fish species were stocked at three similar stocking densities. Low stocking density (LSD): 1.44 kg/m^3 (45 fish/treatment), medium stocking density (MSD): 2.63 kg/m^3 (75 fish/treatment), and high stocking density (HSD): 3.68 kg/m^3 (105 fish/treatment). The stocking densities were carefully chosen to be biologically relevant, aligned with previous research, and safe for fish welfare. They allowed the study to examine performance and immune responses across a realistic range of conditions typical of semi-intensive aquaculture systems.

5.4.6 Determination of growth performance indices

Feed intake, feed conversion ratio, specific growth rate, and weight gain were determined as outlined in chapter 3, section 3.4.7.

5.4.7 Haematological parameters

At the end of the experiment, the determination of haematological variables such as white blood cell count (WBC, $\times 10^3/\text{L}$), haemoglobin concentration (Hgb, g/dL),

haematocrit (Hct, %), red blood cell count (RBC, $\times 10^6/\text{L}$), platelet count ($\times 10^6/\text{L}$), mean corpuscular volume (MCV, fL), mean corpuscular haemoglobin (MCH, g/dL), mean corpuscular haemoglobin concentration (MCHC, g/dL) and blood performance was carried out as described chapter 3, section 3.4.8.

5.4.8 General immune parameters

Lysozyme activity and plasma cortisol were analysed as outlined in chapter 3, under section 3.4.9.

5.4.9 Statistical analysis

Analysis of covariance (ANCOVA) was used to analyse the data to determine the effect of stocking densities on the opercular beat rate, growth parameters (SGR, FCR, and feed intake), haematological parameters (WBC, RBC, HGB, HCT, MCV, MCH, MCHC, platelets, glucose and blood performance), and immunological parameters (lysozyme activity and plasma cortisol) fed with the 0% and 10% *A. afra*-based diet. The slope represents the relationship between the covariate and dependent variables. Normality and homogeneity of variance was confirmed using the Shapiro-Wilk normality test and Levene test, respectively using R studio (version 4.3, 2023).

5.5 RESULTS

5.5.1 The effect of stocking density on growth performance parameters and opercular beat rate

The highest SGR ($3.10 \pm 0.15\%/day$), feed intake (1.75 ± 0.03 g/fish/day), and best FCR (2.85 ± 0.26) in *O. mossambicus* fed D1 were observed at MSD ($P < 0.05$) (Table 5.1). The lowest opercular beat rate (142 ± 4.41 OBR/min) was observed at MSD ($P < 0.05$). In *O. mossambicus* fed D2, the highest SGR ($3.94 \pm 0.17\%/day$), feed intake (1.24 ± 0.26 g/fish/day), and the best FCR (0.68 ± 0.12) were observed at HSD ($P < 0.05$) (Table 5.1). Contrary to fish fed D1 diet, the lowest opercular beat rate (134 ± 2.02) was also recorded at HSD ($P < 0.05$). Fish fed D2 recorded the highest ($P < 0.05$, ANCOVA) IBW, FBW, WG, SGR, and feed intake than in fish fed D1. The opercular beat rate and FCR were significantly higher ($P < 0.05$, ANCOVA) in fish fed D1 than in fish fed D2.

The highest SGR ($3.56 \pm 0.54\%/day$), feed intake (2.26 ± 0.23 g/fish/day), and best FCR (2.42 ± 0.02) in *C. gariepinus* fed D1 were observed at LSD ($P < 0.05$) (Table 5.2). Furthermore, the lowest opercular beat rate (143 ± 4.21 OBR/min) was observed at LSD ($P < 0.05$) (Table 5.2). In *C. gariepinus* fed D2, the highest weight gain (37.98 ± 1.59 g), SGR ($3.63 \pm 1.34 \%/day$), and best FCR (0.80 ± 0.10) were observed at MSD ($P < 0.05$) (Table 5.2). Additionally, the highest feed intake was recorded at MSD (1.55 ± 0.21 g/fish/day), and there was no significant difference ($P > 0.05$) among stocking densities. Contrary to fish fed D1, the lowest opercular beat rate (126 ± 3.48 OBR/min) in fish fed with D2 was observed at MSD (Table 5.2). Growth parameters (IBW, FBW, WG, SGR, and feed intake) were significantly higher ($P < 0.05$, ANCOVA) in fish fed D2 than in fish fed D1 (Table 5.2). The OBR and FCR also followed the same trend as *O. mossambicus* ($P < 0.05$, ANCOVA).

Table 5. 1: Growth performance indices and opercular beat rate of *O. mossambicus* sub-adults (n= 9) reared under different stocking densities fed with the 0% *A. afra*-based diet (D1) and 10% *A. afra*-based diet (D2) after 21 days of feeding.

Growth parameters	Stocking density							
	D1 (0%)				D2 (10%)			
	Low	Medium	High	Slope	Low	Medium	High	Slope
IBW (g)	35.26 ± 1.29	34.53 ± 2.15	35.48 ± 1.32	1.44	36.24 ± 0.26	35.69 ± 0.14	34.24 ± 0.45	3.77
FBW (g)	54.25 ± 2.69	66.14 ± 4.36	60.14 ± 3.11	1.66	62.46 ± 1.26 ^b	76.25 ± 2.25 ^a	78.37 ± 1.47 ^a	2.41
WG (g)	18.99 ± 1.00 ^b	31.61 ± 2.06 ^a	24.66 ± 0.26 ^b	2.83	26.22 ± 2.14 ^b	40.56 ± 4.52 ^a	44.13 ± 3.29 ^a	8.95
SGR (%/day)	2.05 ± 0.03 ^c	3.10 ± 0.15 ^a	2.51 ± 0.24 ^b	0.23	2.59 ± 0.36 ^b	3.61 ± 0.11 ^a	3.94 ± 0.17 ^a	0.67
FCR	4.74 ± 0.67 ^a	2.85 ± 0.26 ^c	3.65 ± 0.13 ^a	-0.55	3.43 ± 0.16 ^a	2.22 ± 0.21 ^b	2.03 ± 0.12 ^c	-0.70
FI (g/fish/day)	0.79 ± 0.02 ^b	1.75 ± 0.03 ^a	1.06 ± 0.12 ^a	0.13	0.43 ± 0.01 ^c	0.64 ± 0.02 ^b	1.24 ± 0.26 ^a	0.41
Survival rate (%)	85.45 ± 1.26 ^b	96.14 ± 2.45 ^a	91.22 ± 0.32 ^a	2.88	100	100	100	0
OBR (OBR/min)	175 ± 2.22 ^c	142 ± 4.41 ^a	152.11 ± 2.14 ^b	-11.5	151 ± 3.22 ^a	147 ± 2.14 ^a	134 ± 2.02 ^b	-8.5

IBW= Initial body weight, FBW = Final body weight, WG = Weight gain, FCR = Food conversion ratio, SGR = Specific growth rate, FI = Feed Intake. In each row, different letters indicate significant differences between groups (P< 0.05). Values are expressed mean ± SD. Low = 45 fish per treatment; Medium = 75 fish per treatment; High = 105 fish per treatment. Different superscripts in a row show a significant difference (P < 0.05, ANCOVA).

Table 5. 2: Growth performance indices and opercular beat rate of *C. gariepinus* sub-adults (n= 9) reared under different stocking densities fed with the 0% *A. afra*-based diet (D1) and 10% *A. afra*-based diet (D2) after 21 days of feeding.

Growth parameters	Stocking density							
	D1 (0%)				D2 (10%)			
	Low	Medium	High	Slope	Low	Medium	High	Slope
IBW (g)	33.40 ± 1.26	35.45 ± 4.21	34.15 ± 1.65	2.55	32.26 ± 0.12	33.28 ± 1.69	31.36 ± 0.54	2.99
FBW (g)	70.56 ± 2.53 ^a	61.26 ± 4.21 ^b	56.21 ± 3.32 ^b	1.35	51.26 ± 2.14 ^b	71.26 ± 6.21 ^a	43.21 ± 3.28 ^b	2.01
WG (g)	37.16 ± 2.14 ^b	25.81 ± 1.00 ^a	22.06 ± 1.52 ^a	-7.55	19.00 ± 1.44 ^c	37.98 ± 1.59 ^a	21.85 ± 2.14 ^b	1.42
SGR (%/day)	3.56 ± 0.54 ^a	2.60 ± 0.21 ^b	2.37 ± 0.65 ^b	-0.60	2.21 ± 0.45 ^c	3.63 ± 1.34 ^a	1.53 ± 0.26 ^b	-0.34
FCR	2.42 ± 0.02 ^c	3.49 ± 0.41 ^b	4.08 ± 0.14 ^a	0.83	1.58 ± 0.61 ^a	0.80 ± 0.10 ^b	1.62 ± 0.12 ^a	0.02
FI (g/fish/day)	2.26 ± 0.23 ^a	2.15 ± 0.32 ^a	1.21 ± 0.41 ^b	-0.53	1.52 ± 0.04	1.55 ± 0.21	1.43 ± 0.37	-0.05
Survival rate (%)	98.42 ± 3.21 ^a	83.14 ± 1.06 ^b	74.41 ± 2.18 ^c	-12.01	100	100	100	0
OBR (OBR/min)	143 ± 4.21 ^b	168 ± 2.34 ^a	171 ± 3.22 ^a	14.00	132 ± 1.01 ^b	126 ± 3.48 ^b	154 ± 1.64 ^a	11

IBW= Initial body weight, FBW = Final body weight, WG = Weight gain, FCR = Food conversion ratio, SGR = Specific growth rate, FI = Feed Intake. Different letters indicate significant differences between groups (P< 0.05) in each row. Values are expressed mean ± SD. Low = 45 fish per treatment; Medium = 75 fish per treatment; High = 105 fish per treatment. Different superscripts in a row show a significant difference (P < 0.05, ANCOVA).

5.5.2 The effect of stocking density on haematological parameters and blood performance

In *O. mossambicus* fed D1, WBC ($10.26 \pm 1.11 \times 10^6/L$), RBC ($4.26 \pm 0.85 \times 10^3/L$), HCT ($28.31 \pm 2.93\%$), HGB (8.46 ± 1.52 g/dL) and blood performance (9.14 ± 2.15) were significantly higher ($P < 0.05$) at MSD (Table 5.3). The MCV (62.48 ± 1.65), MCH (51.52 ± 2.55), MCHC (40.52 ± 3.52), and platelets (36.66 ± 4.65) were significantly higher ($P < 0.05$) at LSD. The highest WBC ($12.53 \pm 0.10 \times 10^6/L$), RBC ($4.82 \pm 0.15 \times 10^3/L$), HCT ($34.38 \pm 1.35\%$) and HGB (8.93 ± 1.69 g/dL) in *O. mossambicus* fed D2 were recorded at HSD ($P < 0.05$) (Table 5.3). Platelets, MCHC, MCV, and MCH all showed the same pattern. The highest ($P < 0.05$) blood performance was also recorded at HSD (9.25 ± 1.45). The highest WBC, RBC, HCT, HGB, MCV, MCH, MCHC, and platelets were recorded highest ($P < 0.05$, ANCOVA) in fish fed D2 than in fish fed D1. Glucose levels were significantly higher ($P < 0.05$, ANCOVA) in fish fed D1 than in fish fed D2 (Table 5.3). The highest ($P < 0.05$, ANCOVA) blood performance was observed in fish fed D2 than in fish fed D1.

Contrary to *O. mossambicus* fed D1, the highest WBC ($7.46 \pm 0.42 \times 10^6/L$), RBC ($3.12 \pm 0.35 \times 10^3/L$), HCT ($25.10 \pm 2.00\%$), HGB (7.21 ± 1.22 g/dL) and blood performance (8.35 ± 0.32) in *C. gariepinus* fed D1 were observed at LSD ($P < 0.05$) (Table 5.4). The MCV, MCH, MCHC, and platelets followed the same trend. Blood performance was also higher at LSD ($P < 0.05$) (Table 5.4). In *C. gariepinus* fed D2, the lowest WBC ($3.86 \pm 1.06 \times 10^6/L$), RBC ($5.56 \pm 0.48 \times 10^3/L$), HCT ($15.43 \pm 1.56\%$), and HGB (5.85 ± 1.47 g/dL) were recorded at HSD ($P < 0.05$) (Table 5.4). The lowest MCV, MCH, MCHC, and platelets were also recorded at HSD. Contrary to *O. mossambicus*, the highest blood performance in *C. gariepinus* was observed at MSD. Haematological parameters (WBC, RBC, HCT, HGB, MCV, MCH, MCHC, and platelets) followed the same trend as *O. mossambicus* ($P < 0.05$, ANCOVA). Glucose and blood performance also followed the same trend as *O. mossambicus* ($P < 0.05 < ANCOVA$).

Table 5. 3: Haematological parameters and blood performance of *O. mossambicus* sub-adults (n = 9) under different stocking densities fed with the 0% *A. afra*-based diet (D1) and 10% *A. afra*-based diet (D2) after 21 days of feeding.

Haematological parameters	Stocking density							
	D1 (0%)				D2 (10%)			
	Low	Medium	High	Slope	Low	Medium	High	Slope
WBCs (10 ³ /L)	5.26 ± 0.36 ^b	10.26 ± 1.11 ^a	6.22 ± 1.48 ^b	1.22	8.48 ± 0.33 ^b	9.52 ± 0.25 ^b	12.53 ± 0.10 ^a	1.59
RBCs (10 ⁶ /L)	1.59 ± 0.21 ^b	4.26 ± 0.85 ^a	2.00 ± 0.25 ^b	3.33	2.75 ± 0.16 ^b	2.78 ± 0.26 ^b	4.82 ± 0.15 ^a	4.06
HCT (%)	20.48 ± 5.65 ^a	28.31 ± 2.93 ^b	22.45 ± 4.22 ^a	1.46	24.91 ± 3.69 ^b	32.40 ± 2.11 ^a	34.38 ± 1.35 ^a	2.44
HGB (g/dL)	4.22 ± 0.26 ^a	8.46 ± 1.52 ^b	5.14 ± 1.11 ^a	1.44	5.63 ± 2.14 ^b	6.29 ± 1.24 ^b	8.93 ± 1.69 ^a	2.00
MCV (fL)	62.48 ± 1.65	60.15 ± 5.26	60.09 ± 4.25	3.31	78.22 ± 4.26 ^a	73.02 ± 5.78 ^a	65.62 ± 2.47 ^b	3.46
MCH (pg)	51.52 ± 2.55	49.24 ± 4.11	48.85 ± 6.27	2.46	45.54 ± 2.14	47.36 ± 1.24	48.80 ± 1.09	2.99
MCHC (g/dL)	40.52 ± 3.52	37.22 ± 4.59	36.15 ± 1.22	2.11	34.27 ± 2.36	34.57 ± 1.39	36.95 ± 1.59	2.56
Platelets (10 ⁶ /L)	36.66 ± 4.65	33.41 ± 2.00	30.75 ± 1.29	3.26	54.66 ± 1.89	56.62 ± 1.39	57.81 ± 1.55	4.21
Glucose (g/dL)	5.21 ± 0.00 ^a	2.54 ± 0.02 ^b	5.00 ± 1.65 ^a	-0.62	5.62 ± 2.11 ^a	4.26 ± 0.14 ^b	3.22 ± 1.26 ^c	0.02
Blood performance	6.58 ± 1.45 ^c	9.14 ± 2.15 ^a	7.27 ± 0.35 ^b	2.39	8.41 ± 0.48 ^b	8.52 ± 2.65 ^b	9.25 ± 1.45 ^a	3.69

In each row, different letters indicate significant differences between groups ($P < 0.05$) RBC: red blood cell counts; HCT: haematocrit; HGB: haemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; WBC: white blood cell counts. Values are expressed mean ± SD. Low = 45 fish per treatment; Medium = 75 fish per treatment; High = 105 fish per treatment. Different superscripts in a row show a significant difference ($P < 0.05$, ANCOVA).

Table 5. 4: Haematological parameters and blood performance of *C. gariepinus* sub-adults (n = 9) under different stocking densities fed with the 0% *A. afra*-based diet (D1) and 10% *A. afra*-based diet (D2) after 21 days of feeding.

Haematological parameters	Stocking density							
	D1 (0%)				D2 (10%)			
	Low	Medium	High	Slope	Low	Medium	High	Slope
WBCs (10 ³ /L)	7.46 ± 0.42 ^a	6.14 ± 1.36 ^{ab}	4.52 ± 0.24 ^b	0.26	6.71 ± 0.26 ^a	6.84 ± 1.36 ^a	3.86 ± 1.06 ^b	1.23
RBCs (10 ⁶ /L)	3.12 ± 0.35 ^a	3.10 ± 0.22 ^a	1.41 ± 0.01 ^b	0.41	13.06 ± 0.11 ^a	12.51 ± 0.39 ^a	5.56 ± 0.48 ^b	3.15
HCT (%)	25.10 ± 2.00 ^b	15.33 ± 5.11 ^a	13.14 ± 1.26 ^a	1.65	31.59 ± 1.33 ^b	26.52 ± 1.29 ^a	15.43 ± 1.56 ^c	2.48
HGB (g/dL)	7.21 ± 1.22 ^b	3.16 ± 0.06 ^a	3.01 ± 0.77 ^a	1.00	9.96 ± 1.28 ^a	8.94 ± 1.36 ^a	5.85 ± 1.47 ^b	2.52
MCV (fL)	51.22 ± 3.16	50.18 ± 1.11	48.21 ± 2.14	4.52	81.36 ± 3.59	81.46 ± 1.74	82.14 ± 1.86	5.09
MCH (pg)	40.22 ± 1.49	38.45 ± 6.21	37.62 ± 1.25	3.06	42.24 ± 4.33	43.26 ± 2.91	43.32 ± 2.14	4.21
MCHC (g/dL)	56.11 ± 2.45	54.42 ± 6.62	51.21 ± 2.65	6.51	32.68 ± 4.49	32.85 ± 2.36	32.93 ± 1.35	7.25
Platelets (10 ⁶ /L)	31.96 ± 1.85	31.15 ± 2.26	30.51 ± 7.14	5.22	58.75 ± 4.36	59.83 ± 1.36	60.39 ± 1.59	5.39
Glucose (g/dL)	2.14 ± 0.25	5.24 ± 1.25	5.66 ± 0.31	0.06	8.21 ± 1.14 ^b	8.69 ± 1.14 ^b	9.04 ± 1.36 ^a	1.05
Blood performance	8.35 ± 0.32 ^a	6.83 ± 1.52 ^b	5.53 ± 0.51 ^c	0.65	8.43 ± 1.93 ^a	8.75 ± 1.94 ^a	6.24 ± 2.14 ^b	1.92

In each row, different letters indicate significant differences between groups (P < 0.05) RBC: red blood cell counts; HCT: haematocrit; HGB: haemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin

concentration; WBC: white blood cell counts. Values are expressed mean \pm SD. Low = 45 fish per treatment; Medium = 75 fish per treatment; High = 105 fish per treatment. Different superscripts in a row show a significant difference ($P < 0.05$, ANCOVA).

5.5.3 Effect of stocking density on immunological parameters

Lysozyme activity in *O. mossambicus* fed with the 0% *A. afra*-based diet was significantly higher ($P < 0.05$) at MSD ($1.24 \pm 0.42 \text{ mL}^{-1}$) (Table 5.5). The highest plasma cortisol was observed at MSD ($5.25 \pm 0.29 \text{ }\mu\text{g/ml}$), and it was significantly different ($P < 0.05$) from LSD and HSD. No significant difference ($P > 0.05$) in plasma cortisol was observed between LSD and HSD (Table 5.5). In *O. mossambicus* fed with the 10% *A. afra*-based diet, the highest lysozyme activity was recorded at HSD ($4.39 \pm 1.32 \text{ mL}^{-1}$), and it was significantly different from LSD and MSD (Table 5.5). No significant difference ($P > 0.05$) in lysozyme activity was observed between LSD and MSD. The lowest plasma cortisol was recorded at HSD ($3.26 \pm 1.03 \text{ }\mu\text{g/ml}$), and it was significantly different ($P < 0.05$) from LSD and HSD. There were significant differences ($P < 0.05$) in plasma cortisol across stocking densities. Lysozyme activity was recorded highest ($P < 0.05$, ANCOVA) in fish fed D2 than in fish fed D1, consequently plasma cortisol was higher ($P < 0.05$, ANCOVA) in fish fed D1 than in fish fed D2 (Table 5.5).

The highest lysozyme activity ($1.54 \pm 0.22 \text{ mL}^{-1}$) in *C. gariepinus* fed with the 0% *A. afra*-based diet was recorded at LSD ($P < 0.05$) (Table 5.6). The lowest plasma cortisol was observed at LSD ($3.42 \pm 0.25 \text{ }\mu\text{g/ml}$), and it was significantly different ($P < 0.05$) from MSD and HSD. However, no significant differences ($P > 0.05$) in plasma cortisol were observed between MSD and HSD (Table 5.6). In *C. gariepinus*, the lowest lysozyme activity was recorded at HSD ($2.22 \pm 0.11 \text{ mL}^{-1}$), and it was significantly different from LSD and MSD (Table 5.6). No significant difference ($P > 0.05$) in lysozyme activity was observed between LSD and MSD. The lowest plasma cortisol was observed at MSD ($5.34 \pm 1.03 \text{ }\mu\text{g/ml}$), and it was significantly different ($P < 0.05$) from HSD (Table 5.6). No significant difference ($P > 0.05$) in plasma cortisol was observed between LSD and MSD. Fish fed D2 recorded the highest lysozyme activity than in fish fed D1 ($P < 0.05$, ANCOVA). In plasma cortisol, fish fed D2 recorded the lowest ($P < 0.05$, ANCOVA) than in fish fed D1.

All the results of this chapter are summarized in Table 5.7 and 5.8 for *O. mossambicus* and *C. gariepinus*, respectively.

Table 5. 5: Effect of different stocking densities on the immunological parameters of *O. mossambicus* sub-adults (n= 9) fed with the 0% *A. afra*-based diet (D1) and 10% *A. afra*-based diet (D2) after 21 days of feeding.

Immunological parameters	Stocking density							
	D1 (0%)				D2 (10%)			
	Low	Medium	High	Slope	Low	Medium	High	Slope
Lysozyme activity	0.21 ± 0.00 ^a	1.24 ± 0.42 ^b	0.56 ± 0.01 ^a	-0.74	2.36 ± 0.32 ^b	2.41 ± 0.14 ^b	4.39 ± 1.32 ^a	0.26
Plasma cortisol	9.51 ± 3.41 ^b	5.25 ± 0.29 ^a	10.26 ± 1.24 ^b	1.26	8.16 ± 2.14 ^a	5.24 ± 1.24 ^b	3.26 ± 1.03 ^c	1.00

In each row, different letters indicate significant differences between groups ($P < 0.05$, ANCOVA). Values are expressed mean ± SD. Low = 45 fish per treatment; Medium = 75 fish per treatment; High = 105 fish per treatment.

Table 5. 6: Effect of different stocking densities on the immunological parameters of *C. gariepinus* sub-adults (n= 9) fed with the 0% *A. afra*-based diet (D1) and 10% *A. afra*-based diet (D2) after 21 days of feeding.

Immunological parameters	Stocking density							
	D1 (0%)				D2 (10%)			
	Low	Medium	High	Slope	Low	Medium	High	Slope
Lysozyme activity	1.54 ± 0.22 ^a	0.75 ± 0.04 ^b	0.50 ± 0.02 ^b	-0.52	3.14 ± 0.31 ^a	3.26 ± 0.74 ^a	2.22 ± 0.11 ^b	1.11
Plasma cortisol	3.42 ± 0.25 ^a	6.54 ± 0.22 ^b	6.65 ± 0.19 ^b	2.11	7.26 ± 1.32 ^a	5.34 ± 1.03 ^b	9.36 ± 3.33 ^a	1.02

In each row, different letters indicate significant differences between groups ($P < 0.05$, ANCOVA). Values are expressed mean ± SD. Low = 45 fish per treatment; Medium = 75 fish per treatment; High = 105 fish per treatment.

Table 5. 7: Summary table of the results in *Oreochromis mossambicus*

Parameters	Relationship between D1 and D2
Opercular beat rate	The lowest OBR in D1 was observed at MSD, whereas D2 showed the lowest at HSD.
Specific growth rate	D1 recorded the highest SGR at MSD, whereas D2 exhibited the highest at HSD.
Feed conversion ratio	While D2 recorded the best FCR at HSD, D1 had the best FCR at MSD.
Feed intake	At MSD, D1 had the highest feed intake, while at HSD, D2 had the highest.
Survival rate	Fish fed D1 had an increase in mortality rate as stocking densities increased, while D2 had no mortalities.
WBC	While D2 had the highest WBC at HSD, D1 had the highest at MSD.
RBC	D1 had the highest RBC levels at MSD, while D2 had the highest at HSD.
HCT	D1 had the highest HCT levels at MSD, while D2 had the highest at HSD.
HGB	At HSD, D2 had the highest HGB, while D1 had the highest at MSD.
MCV	While D2 had the highest MCV at HSD, D1 had the highest at MSD.
MCH	While D2 had the highest MCH at HSD, D1 had the highest at MSD.
MCHC	D1 had the highest MCHC levels at MSD, while D2 had the highest at HSD.
Platelets	D1 recorded the highest platelets at MSD, whereas D2 exhibited the highest at HSD.
Glucose	The lowest glucose in D1 was observed at MSD, whereas D2 showed the lowest at HSD.
Blood performance	D1 had the highest blood performance levels at MSD, while D2 had the highest at HSD.
Lysozyme activity	At MSD, D1 had the highest lysozyme activity, while at HSD, D2 had the highest.

Plasma cortisol	Even though D2 exhibited the lowest plasma cortisol at HSD, D1 had the lowest at MSD.
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Table 5. 8: Summary table of results in *Clarias gariepinus*

Parameters	Relationship between D1 and D2
Opercular beat rate	D2 demonstrated the lowest OBR at MSD, while D1 had the lowest OBR at LSD.
Specific growth rate	At LSD, D1 had the highest SGR levels, whereas at MSD, D2 had the highest.
Feed conversion ratio	D1 had the highest FCR at LSD, but D2 had the highest FCR at MSD.
Feed intake	D1 had the highest feed intake levels at LSD, while D2 had the highest at MSD.
Survival rate	Mortality rate in D1 increased with an increase in stocking densities while D2 had no mortality.
WBC	D1 had the highest WBC levels at LSD, while D2 had the highest at MSD.
RBC	D1 recorded the highest RBC at LSD, whereas D2 exhibited the highest at MSD.
HCT	While D2 had the highest HCT at MSD, D1 had the highest at LSD
HGB	At LSD, D1 had the highest HGB, while at MSD, D2 had the highest.
MCV	While D2 had the highest MCV at MSD, D1 had the highest at LSD
MCH	D1 recorded the highest MCHC at LSD, whereas D2 exhibited the highest at MSD.
MCHC	D1 had the highest MCHC levels at LSD, while D2 had the highest at MSD.
Platelets	At MSD, D1 had the highest platelets, while at HSD, D2 had the highest.
Glucose	D2 demonstrated the lowest glucose at MSD, while D1 had the lowest at LSD.

Blood performance	D1 had the highest blood performance levels at LSD, while D2 had the highest at MSD.
Lysozyme activity	D1 recorded the highest lysozyme activity at LSD, whereas D2 exhibited the highest at MSD.
Plasma cortisol	D1 had the lowest glucose at LSD, while D2 had the lowest at MSD.

5.6 DISCUSSION

The lowest opercular beat rate in *O. mossambicus* fed with the 0% *A. afra*-based diet were observed at medium stocking density (2.63 kg/m³). At medium stocking density, little to no stress is anticipated that could result from inadequate space or high competition for food. The fish might, therefore, be able to channel their energy on growing rather than competing for food or resources. However, in *C. gariepinus* fed with the 0% *A. afra*, the opercular beat rate increased with increasing stocking density. The highest opercular beat rate was observed at medium (2.63 kg/m³) and high stocking density (3.68 kg/m³). This might indicate that medium stocking density (2.63 kg/m³) and high stocking density (3.68 kg/m³) stressed *C. gariepinus* fed with the 0% *A. afra*-based diet. Additionally, this may indicate that the stocking density of 3.68 kg/m³ stressed both tilapia and catfish thus led to poor growth performance due to increased competition for resources such as food and space. In *O. mossambicus* fed with the 10% *A. afra*-based diet, the opercular beat rate decreased with stocking density throughout the experiment. At high stocking density, oxygen levels in the water might be lower due to the cumulative respiration of the fish fed with the 10% *A. afra*-based diet. Thus, it reduces the opercular beat rate as an adaptive response to conserve energy and manage oxygen use more efficiently. However, in *C. gariepinus* fed with the 10% *A. afra*, the lowest opercular beat rate was observed at medium stocking densities (2.63 kg/m³). *Clarias gariepinus* experience less competition for oxygen, allowing them to breathe more efficiently (Mbanga *et al.*, 2018), and their opercular beat rate is lower because there is sufficient oxygen available in the water to meet their oxygen needs, which might have led to low opercular beat rate. Freccia *et al.* (2021) and Ferreira *et al.* (2021) indicated that stocking densities can affect the opercular beat rate fed with commercial diets.

In both fish species with 0% *A. afra*, feed intake declined with increased stocking density. This indicates that stocking density of 3.68 kg/m³ stressed both fish species and thus led to poor growth performance due to increased competition of resources as mentioned in opercular beat rate. Stress may trigger fish to strive harder to obtain oxygen, and food which reduces their available energy for growth and can result in increased opercular beat rates. Several studies have shown that tilapia and catfish fed

control or commercial diets resulted in reduced growth performance at high stocking densities of 3.14 to 5.58 kg/m³ (Tiimub *et al.*, 2020; Abdel-Ghany *et al.*, 2021). However, in *O. mossambicus* fed 10% *A. afra*-based diet, the best feed intake was observed at high stocking density. The highest growth performance in fish stocked at high density (3.68 kg/m³) suggests that the 10% *A. afra*-based diet helped in maximizing feed intake and SGR while minimizing the effects of stress, and fish were able to convert feed into body mass efficiently, leading to optimal growth. Verdal *et al.* (2018) and Rahman *et al.* (2021) reported that tilapia can have the best feed intake at high densities due to increased feed efficiency and reduced energy expenditure from lower activity levels. In contrast to this study, Gibtan *et al.* (2008), Wu *et al.* (2023), and Al-Zahrani *et al.* (2023) recorded the best feed intake at low stocking density (1.26 kg/m³). On the contrary, the best feed intake in *C. gariepinus* fed with the 10% *A. afra* was observed at a stocking density of 2.63 kg/m³ (MSD). These results are consistent with opercular beat rate observations. Although *C. gariepinus* is known to tolerate very high stocking densities up to 200 kg/m³ in intensive systems (Hecht *et al.*, 1996). However, the highest density used in this study was 3.65 kg/m³. Despite operating well below the species' maximum tolerance, fish at this density showed reduced growth performance and physiological response compared to those at medium stocking density (MSD). However, Wu *et al.* (2018) also reported that *C. gariepinus* are more territorial and aggressive, especially in crowded conditions, which can lead to increased stress, suppressed appetite, and competition of food. Therefore, the results observed in this study suggest that *C. gariepinus* recorded better oxygen levels at medium density than at high density. This highlights the importance of identifying system-specific optimal densities rather than relying solely on reported maximum tolerance.

Specific growth rate in both fish species fed with the 0% *A. afra* also declined with increasing stocking density. This is attributed to the reduced feed intake as observed. This observation agrees with the studies conducted by Nwipie *et al.* (2015) and Oké and Goosen (2019), who recorded a decline in SGR of *C. gariepinus* at low stocking density of 1.02 kg/m³. These results are consistent with the results obtained FCR. However, the supplementation of 10% *A. afra* in the diets of *O. mossambicus* recorded a higher SGR, feed intake and the best FCR at higher densities. *Clarias gariepinus*

fed with 10% *A. afra* recorded a higher SGR, feed intake and the best FCR at medium densities. These results are consistent with the observations in opercular beat rate. It seems from the results that *A. afra* helped the fish to adapt, conserve energy and manage oxygen more efficiently. While high stocking densities are often necessary in catfish culture to reduce cannibalism, particularly during early developmental stages; excessively high densities can lead to negative impacts on growth performance, water quality, and immune function. Therefore, stocking density must be carefully optimized to balance the reduction of cannibalism with the maintenance of physiological health and growth. Moderately high densities may provide a compromise by minimizing cannibalism without excessively compromising innate immunity and growth.

In both fish species fed with the 0% *A. afra*-based diet, mortality rate increased with stocking density, and this correlates with several studies conducted (Oké and Goosen, 2019; Abdel-Aziz *et al.*, 2021; Li *et al.*, 2021; Komal *et al.*, 2024). However, no mortality was observed in both fish species fed with the 10% *A. afra*-based diet across densities. This may indicate the potential effect of using the 10% *A. afra*-based diet on reducing stress in both fish species. Additionally, this is consistent with the results obtained from previous chapters (3 and 4), wherein both fish species fed 10% *A. afra* were subjected to stress, and no mortality was recorded. This indicates that the 10% *A. afra*-based diet managed to boost the fish's immune response, making them more resilient to stress caused by stocking densities that could have led to mortality.

In this study, the observed values for Specific Growth Rate (SGR < 2 %/day), feed intake (< 2 g/fish/day), and Feed Conversion Ratio (FCR > 3) did not fall within the optimal ranges typically reported for *Clarias gariepinus* reared in RAS. Previous studies have shown that under well-optimized conditions, *C. gariepinus* and *O. mossambicus* generally exhibits SGR values above 2–3 %/day and FCR values below 2.0 (Adewolu and Adoti, 2010; Solomon and Udoji, 2011). These results are consistent with the results of chapter 3 and 4. The suboptimal performance observed in the current study may be attributed to system-related factors such as limited water exchange rates, suboptimal temperature control (ambient/tunnel temperature not recorded), or physiological stress at higher stocking densities. These findings

underscore the importance of monitoring system conditions and environmental variables in RAS to ensure optimal growth and feed utilization in *O. mossambicus* and *C. gariepinus*.

Oreochromis mossambicus stocked at medium density and high density showed a higher plasma cortisol and glucose when fed with the 0% *A. afra*-based diet. High stocking density is known to increase stress levels in fish. This may indicate that the 0% *A. afra*-based diet failed in reducing both plasma cortisol and glucose levels when stocked at a density of 2.63 kg/m³ and 3.68 kg/m³. On the other hand, the lowest plasma cortisol and glucose in *O. mossambicus* fed with the 10% *A. afra*-based diet were observed at high stocking density. This shows that 10% *A. afra*-based diet reduced physiological stress, leading to lower cortisol and glucose levels. Similar to these observations, Shourbela *et al.* (2017) observed the lowest plasma cortisol and glucose levels in *O. niloticus* stocked at a high density of 3.36 kg/m³. In contrast, Nuwansi *et al.* (2021) observed the highest plasma cortisol and glucose levels in *O. niloticus* stocked at the high-density group of 5.59 kg/m³. *Clarias gariepinus* plasma cortisol and glucose increased with stocking densities in fish fed with the 0% *A. afra*-based diet. This suggest that medium and high stocking densities stressed *C. gariepinus* fed 0% *A. afra*. Abdel-Hamid (2018) and Shoko *et al.* (2016) observed the highest cortisol and glucose levels at a medium density of 2.14 kg/m³ and a higher stocking density of 4.26 kg/m³. This might have been a result of crowding stress. However, the lowest plasma cortisol and glucose levels observed in *C. gariepinus* fed with the 10% *A. afra*-based diet at medium stocking density (2.63 kg/m³) may indicate that *C. gariepinus* were not stressed.

As mentioned in previous chapters, haematological parameters are not good indicators of stress in fish because they give contradictory results. The highest blood performance in *O. mossambicus* fed with the 10% *A. afra*-based diet was observed at high stocking density (3.68 kg/m³). On the contrary, the highest blood performance in *O. mossambicus* fed with the 0% *A. afra*-based diet was observed at medium stocking density (2.63 kg/m³). This can be attributed to the tilapia's response to stress when fed with the 10% *A. afra*-based diet and this shows that fish were able to adjust their blood

performance to maintain efficient circulation and oxygen delivery. This adjustment assists them to cope with the physiological demands of high stocking density. The highest blood performance in *C. gariepinus* fed with the 0% *A. afra*-based diet was observed at low stocking density (1.44 kg/m³), whereas the highest blood performance in fish fed with the 10% *A. afra*-based diet was observed at medium stocking density (2.63 kg/m³). This shows the potential effect of the 10% *A. afra*-based diet in improving the health status of the fish at medium stocking density compared to fish fed with the 0% *A. afra*-based diet. However, there was low blood performance in *C. gariepinus* at high stocking density (3.68 kg/m³). This suggests that *C. gariepinus* has concentrated on conserving energy and resources, which led to a lower blood performance at high stocking density. This demonstrates that blood performance can give reliable results, and it can be used to monitor stress levels in fish. Furthermore, these findings likely suggest that, when both species are stocked at different densities, the 10% *A. afra*-based diet may have elicited superior health responses.

Several studies have indicated that tilapia stocked at a density of 3.02 to 6.14 kg/m³ resulted in a decline in lysozyme activity and increased stress levels (Abdel-Tawwab *et al.*, 2014; Telli *et al.*, 2014). However, the highest lysozyme activity in *O. mossambicus* fed with the 10% *A. afra*-based diet was observed at the stocking density of 3.68 kg/m³, while *O. mossambicus* fed with the 0% *A. afra*-based diet recorded the highest lysozyme activity at the stocking density of 2.63 kg/m³. This may indicate that *O. mossambicus* have a better immune response when they are fed with the 10% *A. afra*-based diet and stocked at a density of 3.68 kg/m³. In *C. gariepinus* fed with the 10% *A. afra*-based diet, the highest lysozyme activity was found at a stocking density of 2.63 kg/m³, while *C. gariepinus* fed with the 0% *A. afra*-based diet showed the highest lysozyme activity at the stocking density of 1.44 kg/m³. These results also indicate that *C. gariepinus* have a better immune response when they are fed with the 10% *A. afra*-based diet and reared at a density of 2.63 kg/m³. Yin *et al.* (2009) stated that an increase in lysozyme levels indicates enhanced phagocytic activity. This is confirmed by the results in this study, and it suggests that 10% *A. afra*-based diet may have contributed to a more robust immune response of *O. mossambicus*, resulting in elevated lysozyme activity to combat stressors such as increased competition and elevated waste levels at high stocking density. *Clarias*

gariepinus may rely more on different components of their immune system, such as anti-body mediated immunity or other enzymes, rather than significantly regulating lysozyme activity under stress. Nwipie *et al.* (2015) reported the highest lysozyme activity in *C gariepinus* stocked at a medium density of 2.02 kg/m³. *Clarias gariepinus* was stocked at high density and recorded the lowest lysozyme activity. A similar observation was made by Chen *et al.* (2012), Refaey *et al.* (2024), and Scherer *et al.* (2023).

Conclusion

The opercular beat rate, growth performance, and innate immunity of both species fed with the 0% *A. afra* were affected by stocking densities. However, the best growth of *O. mossambicus* in terms of SGR, FCR, and feed intake fed with the 10% *A. afra*-based diet was found at high stocking density (3.68 kg/m³) fed with the 10% *A. afra*-based diet. The best opercular beat rate, glucose, and blood performance were observed at the highest stocking density of 3.68 kg/m³ in fish fed with 10% *A. afra*. Additionally, the best plasma cortisol and lysozyme activity were observed at the stocking density of 3.68 kg/m³. This shows that *O. mossambicus* has a better immune response when stocked at HSD (3.68 kg/m³) while supplemented with the 10% *A. afra*-based diet. In *C. gariepinus*, the best growth was observed at a medium stocking density of 2.63 kg/m³ when fed with 10% *A. afra*. The best opercular beat rate, glucose, blood performance, plasma cortisol, and lysozyme activity were also found at a stocking density of 2.63 kg/m³. A medium stocking density of 2.63 kg/m³ resulted in reduced fish stress resulting from competition for food and space, allowing better food consumption and utilization and leading to better growth performance in *C. gariepinus*. This study recommends that tilapia can be reared at a density of 3.68 kg/m³ and catfish at a density of 2.63 kg/m³ and fed with the 10% *A. afra*-based diet on their diets for optimal growth performance and innate immunity. Stocking density did not affect the opercular beat rate, growth performance, and innate immunity in *O. mossambicus* fed with the 10% *A. afra*-based diet. In *C. gariepinus* fed with the 10% *A. afra*-based diet, stocking density also did not affect the opercular beat rate, growth performance, and innate immunity; high stocking density resulted in reduced fish growth. Thus, the null hypothesis is accepted.

CHAPTER 6

**GENERAL DISCUSSION,
RECOMMENDATIONS AND
CONCLUSION**

CHAPTER 6: GENERAL DISCUSSION, RECOMMENDATIONS AND CONCLUSION

In recent years, the use of medicinal plants has received growing attention as a sustainable feed additive for aquafeed production (Van Hai, 2015). Several studies have used medicinal plants to promote growth performance and disease resistance in different fish species. This dissertation addressed the potential use of the *Artemisia afra* essential oil in the diets of *O. mossambicus* and *C. gariepinus* when subjected to various stressors.

The effect of handling stress in both fish species fed with *A. afra*-based diets

This study showed that handling stress affected the opercular beat rate, growth performance, and innate immunity of both freshwater fish species fed with the 0% *A. afra*-based diet. On the other hand, the opercular beat rate, growth performance, and innate immunity of *O. mossambicus* and *C. gariepinus* fed with the 10% *A. afra*-based diet were not affected by handling stress. This indicates that *A. afra* oil possesses compounds that assist the fish in resisting stress during handling. This study will help fish farmers with poor fish husbandry practices to handle the fish without affecting the growth and immunity of the fish when fed with the 10% *A. afra*-based diet. However, this study did not investigate the effect of handling stress on fish mucosal health. This would have assisted in understanding the effect of handling stress on fish skin, gills, and gastrointestinal tract in fish fed with *A. afra*-based diets. The mucosal surfaces are the primary barrier between the fish and pathogens. Fernandez *et al.* (2024) reported that handling can cause physical damage to these surfaces, disrupting the mucus layer and making fish more susceptible to infection. Fish mucus helps to protect fish from pathogens and parasites (Dawar *et al.*, 2024). Therefore, a more comprehensive study should accurately assess the extent to which handling stress compromises fish mucosal health and ways to mitigate these effects using any plant of choice.

The effect of feed deprivation in both fish species fed with *A. afra*-based diets

The findings from the handling stress are supported by the observations from the feed deprivation experiment. For example, opercular beat rate, growth performance, and innate immunity were all affected when both fish (deprived) were fed with the 0% *A. afra*-based diet. However, these parameters were not affected in fish fed with the 10% *A. afra*-based diet across feed deprivation periods. This is evidence that both fish species' health improved as a result of the inclusion of the *A. afra* essential oil in the diet. It is important to note that when depriving fish of feed, it will grow to a certain point, inevitably reducing the feed cost. The limitation in the feed deprivation period lies in the balance between growth and reduced feed cost. Therefore, their balance depends on finding the appropriate feed deprivation period. For instance, if feed deprivation is too long, it can cause lasting stunted growth and leads to increased fish mortality (Syanya *et al.*, 2023). However, this study shows that if properly managed, a short-term feed deprivation period can be managed by supplementing diets with 10% *A. afra*-based diet. This study also showed that *A. afra* can reduce costs without negatively affecting the growth of the fish. Therefore, the balance lies in managing the optimal deprivation-feeding period to maximize compensatory growth and reduce feed costs without affecting the health of the fish. The Mozambique tilapia and African catfish have different feed deprivation periods. This is because they respond differently to feed-deprivation periods (Liu *et al.*, 2013; Meule, 2020). It is thus advisable for farmers to have separate feed deprivation periods for each fish species. Moreover, these periods should differ for age and fish size between species. Further studies should thus focus on the effect of feed deprivation periods on fish of the same size, species, and age when fed with diets supplemented with any medicinal plant of choice.

The effect of rearing both species at different stocking densities and supplemented with *A. afra*-based diets

It is in the interests of any farmer to increase profit by stocking fish in higher densities. However, increasing stocking density can lead to poor water quality and increased stress, leading to poor growth rates and decreased innate immunity, which makes fish susceptible to diseases. This study observed that fish fed with the 0% *A. afra*-based diet while subjected to different stocking densities had poor growth performance and reduced innate immunity. This is consistent with the findings observed in the handling

stress and feed deprivation experiments, which both showed that fish in these groups were severely stressed; thus, growth performance and innate immunity were compromised. In contrast, the growth performance and innate immunity of fish fed with the 10% *A. afra*-based diet were not affected, regardless of the stocking density. This demonstrates the efficiency of the *A. afra* essential oil in reducing stress and improving the growth and immunity of both fish species. Kemper (2023) reported that at high stocking densities, there is improved growth when fish are accustomed to crowded environments, thus behaving in a less hostile manner towards each other. A high stocking density of 3.68 kg/m³ is recommended in this study for rearing tilapia. In catfish, low stocking density reduced the growth performance. This study shows that medium stocking densities of 2.63 kg/m³ are recommended for rearing catfish when fed with the 10% *A. afra*-based diet. This shows that fish species can also be affected by stocking density. Thus, the limitation of this experiment is species-specific stocking density. In this study, different fish species were stocked in similar stocking densities. Tilapia and catfish have different environmental conditions, growth rates, and behaviours. At high densities, tilapia generally perform better than catfish, mainly due to their adaptability to crowded conditions, higher tolerance for lower oxygen, and resilience against stress (Holhorea *et al.*, 2024; Minahal *et al.*, 2024). Hence, tilapia performed better than catfish in this study when stocked at densities of 1.44, 2.63, and 3.68 kg/m³. Therefore, each species should have had its stocking density. Stocking densities used in this should have been used for tilapia only, while catfish stocking densities should have been lower than those used for tilapia.

Overall limitations and implications of this study

Overall, this study showed that *A. afra* essential oil reduced stress in *O. mossambicus* and *C. gariepinus*, which were subjected to various stressors. However, the limitation of this study was the use of haematological parameters as a fish health monitoring tool. Haematological parameters are not reliable because they are easily affected by farming activities such as handling, feed deprivation, and stocking density. A new technique called blood performance (BP) has been recently introduced and was adopted in this study as a replacement or backup for the haematological data to produce reliable and conclusive results. This technique incorporates four standard

haematological parameters, namely white blood cells, red blood cells, haemoglobin cells, and haematocrit counts. The idea behind this formula is that any single component of this formula cannot be reliable enough as a biomarker of fish health. Various aquaculture studies have measured the following components: red blood cells, white blood cells, haemoglobin, and haematocrit. However, because these parameters do not always follow the same trend across experimental fish, it is difficult to draw a firm conclusion about which parameter should be considered (Esmaeili, 2021). Therefore, BP as a new formula was introduced, which is a more reliable indicator of fish health when it is compared between groups in the same experiment or farm. In this study, BP showed that *A. afra* essential oil reduced stress in both fish species subjected to handling stress, feed deprivation periods, and stocking density. These results were consistent with growth performance and immunological parameters. This provides evidence that BP is a good indicator for monitoring fish health, and this study recommends further studies to use it to monitor fish health. The BP also confirms the results from the handling, feed deprivation, and stocking density experiments that using the *A. afra*-based diets reduces stress in fish.

Another limitation of this study was using a completely randomized design (CRD) instead of using a 3x3x3 factorial design. Factorial designs help in controlling variables across treatment groups, which can improve the reliability of results. With CRD, randomization across multiple factors may not adequately control variability, especially when the factors have complex interdependencies. It is, therefore, advisable that the handling times, feed deprivation, and stocking density should have been evaluated concurrently. However, due to limitations of resources, it was practically impossible to do a 3x3x3 factorial design.

CONCLUSION

The current study showed the potential of using *A. afra* essential oil in the diets of *O. mossambicus* and *C. gariepinus* when subjected to poor fish husbandry practices. Handling stress and feed deprivation periods affected the growth performance and innate immunity of *O. mossambicus* and *C. gariepinus* when the fish were fed with the 0% *A. afra*-based diet. In contrast, both fish species fed the 10% *A. afra*-based diet

were not affected by handling stress and feed deprivation periods. A high stocking density of 3.68 kg/m³ is recommended for rearing *O. mossambicus*, whereas a medium stocking density of 2.63 kg/m³ is recommended for rearing *C. gariepinus* fed with the 10% *A. afra*-based diet at the farm level. The lysozyme activity was not influenced by the fish husbandry practices in both fish species fed with the 10% *A. afra*-based diet. It was noted that *C. gariepinus* had a higher growth performance and immunity than *O. mossambicus* when handled and deprived of feed. However, the case was different in stocking density. The results obtained in this study indicate that stress in cultured *O. mossambicus* and *C. gariepinus* could be reduced by feeding the fish with diets supplemented with 10% *A. afra*-based diet essential oil.

SIGNIFICANCE OF THE STUDY

Due to stress and poor husbandry practices, tilapia and catfish farmers face several challenges. Therefore, this study advises farmers to use the *A. afra* essential oils in fish diets to reduce stress. Farmers can use the *A. afra*-based diet because they are readily available, cheap, and easy to apply. The plant contains compounds such as immunostimulatory, antibacterial, and antistress properties, which offer protection against many diseases and stress in fish. This can help farmers improve revenues in their farms because fish will not be stressed when handled, deprived of feed, and stocked at higher densities.

RECOMMENDATIONS

It is recommended that further studies be conducted on blood performance as a tool for monitoring fish health. The stocking density should be reduced to maximize the growth and innate immunity of catfish at high densities. This study also recommends that future studies be undertaken to evaluate the effect of climate change on farmed *O. mossambicus* and *C. gariepinus* fed with *A. afra*-based diets or any medicinal plant of choice.

CHAPTER 7

REFERENCES

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