Evaluation of Water Fern (*Salvinia molesta*) as an Alternative Source of Omega-3 Polyunsaturated Fatty Acids for Cultured Tilapia

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Centre for Biotechnology and Bioinformatics

Declaration

This thesis contains no material which has been accepted for award of a degree or diploma by any other tertiary institution. To the best of my knowledge the thesis contains no material written or published by another person, except where due reference is made.

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ABSTRACT

Long chain omega-3 PUFAs; EPA and DHA found mainly in fish, have been reported to have cardio-protective and other health benefits in humans. Unfortunately, capture fisheries cannot meet the demand for these fatty acids. As a result aquaculture has been on the increase with tilapia being one of the most cultured fishes. Recent studies however, have reported the presence of harmful combination of fatty acid (high n-6: n-3) in cultured tilapia as a result of inclusion of plant oil high in omega-6 in their diet. To achieve a low n-6: n-3 ratio, the aquaculture industry has shifted its attention to identifying alternative sources of n-3 for inclusion in fish feeds.

The present study was conducted to evaluate *Salvinia molesta* an invasive aquatic weed as an alternative source of omega-3 for cultured tilapia. In triplicate groups of 100, tilapia fingerlings weighing 0.35 ± 0.03g (mean ±SE) were fed on either commercial tilapia fingerling feed only, or commercial feed supplemented with *S. molesta* at 10, 20 or 30 %, for a period of 56 days. Lipids were extracted from feed and the fish fillet using the Folch method and fatty acid profile analyzed by gas chromatography.

Results from this study showed that inclusion of *S. molesta* in the experimental diets had no significant impact on fish growth ($p \leq 0.05$). However, the concentration of n-3 in fish oil extracted from fish fillet increased as the level of *S. molesta* in diet increased (0.71 to 1.45mg/g of feed, control & 30% *S. molesta* diet respectively). This also caused an overall decrease in the n-6: n-3 ratio in fillet from 6:1 in fish fed commercial diet only to 4:1 in fish receiving 30% *S. molesta*.

Although supplementation of commercial feed with *S. molesta* increased the amount of n-3 in the experimental fish diet and the fillet, the effect was however minimized by the large amount of linoleic acid (n-6) in the commercial diet. Results from this study provide evidence that *S. molesta* is a good and potential source of n-3 for cultured tilapia that can be utilized at industrial scale.

Dedication

To my mother, Phane Kwamboka

Who has sacrificed much to ensure I get a decent education

Acknowledgement

First and foremost I would like to thank my father in heaven for giving me life, health and ability to study and carry out this research. Thank you for granting me an opportunity to get education and for bringing me across such wonderful people, who guided and even financed this research.

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To all my friends who have contributed to my success in one way or another, may God bless you immensely.

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List of Abbreviations

12-HETE:	12-Hydroxyeicosatetraenoic Acid
15-HETE:	15(*S*)-Hydroxyeicosatetraenoic Acid
5-HETE:	5-Hydroxyeicosatetraenoic Acid
ALA:	Alpha linolenic acid
ARA:	Arachidonic acid
CAD:	Coronary Artery Disease
CHD:	Coronary Heart Disease
COX:	Cyclooxygenase
DGLA:	Dihomo-Gamma Linolenic Acid
DHA:	Docosahexaenoic Acid
EPA:	Eicosapentaenoic Acid
ESP:	Economic Stimulus Programme
FA:	Fatty Acids
FAMEs:	Fatty Acid Methyl Esters
GLA:	Gamma Linolenic Acid
HEPE:	Hydroxyeicosapentaenoic Acid
HODE:	Hydroxyoctadecadienoic Acid
HPEPE:	Hydroperoxyeicosapentaenoic Acid

HPETE:	Hydroperoxyeicosatetraenoic Acid
LA:	Linoleic acid
LC:	Long Chain
LDL:	Low Density Lipoprotein
LOX:	Lipoxygenase
LT:	Leukotriene
PG:	Prostaglandin
PLA2:	Phospholipase A_2
PUFA:	Polyunsaturated Fatty Acids
SDA:	Stearidonic Acid
TX:	Thromboxane
VLDL:	Very Low Density Lipoprotein
ω-3 (n-3):	Omega 3
ω-6 (n-6):	Omega 6

CHAPTER ONE

1.0. INTRODUCTION

Vertebrates including humans and fish require proteins, carbohydrates, lipids as well as vitamins and minerals for normal growth, development and reproduction (Gibney, Vorster, & Kok, 2002; Mjoun, Rosentrater, & Brown, 2010). Proteins are required for body building and synthesis of functional macro-molecules; while carbohydrates are chiefly involved in meeting the body's energy demands. Lipids, on the other hand, are involved in a myriad of functions which include synthesis of cell membrane components; providing thermal cushion from exogenous and endogenous heat shock; energy storage; protection of delicate internal body organs and acting as precursors of hormones and signaling molecules (Lehninger, Nelson, & Cox, 2005).

Lipids are a diverse group of naturally occurring organic compounds that are soluble in organic solvents such as acetone, petroleum and ether, and insoluble or partially soluble in water (Owusu-Apenten, 2005). They include waxes, sterols, phospholipids, triacylglycerols and fat soluble vitamins, each of which play different functional roles in the body. Lipids are mainly composed of fatty acids which are carboxylic acids with hydrocarbon chains ranging from 4-36 carbons long. Fatty acids can either be fully saturated (having no double bond on their hydrocarbon chain), monounsaturated or polyunsaturated. The length and degree of unsaturation (number of double bonds) of the hydrocarbon chain largely determines the melting point and solubility of the fatty acids and the compounds that contain them (Lehninger, et al., 2005).

Omega-3 (ω-3) fatty acids are a class of polyunsaturated fatty acids (PUFAs) with their first double bond on the third carbon from the methyl end of the hydrocarbon chain (Nettleton, 1995). These fatty acids cannot be synthesized in the human body *de novo* and hence have to be supplied in the diet (Roos, Mavrommatis, & Brouwer, 2009). They include the short chain (SC) omega-3; α-linolenic acid (C18:3n-3) and long chain (LC) omega-3; eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22:6n-3) among others. In addition to being required for normal growth and development,

eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been associated with a number of health benefits including primary and secondary prevention of coronary heart disease and inflammation in humans (Connor, 2000; Riediger, Othman, Suh, & Moghadasian, 2009).

Fish and marine animals are the main dietary sources of long chain omega-3 PUFAs in nature (Karapanagiotidis, Bell, Little, & Yakupitiyage, 2007) and fish form a major component of the human diet. Total production from wild fisheries seem to have reached a plateau at about 90 million tons, annually (FAO, 2010). Capture fisheries from lakes, rivers and oceans will therefore not be able to meet the rising demand for fish which is expected to rise as the human population grows and people become more aware of the health benefits of fish consumption. To fill the gap between demand and supply, aquaculture has been on the rise, with total production from aquaculture increasing from 41.9 million metric tons in 2004 to 55.1 million metric tons in 2009 accounting for 46% of total food fish produced (FAO, 2010).

Tilapia is a freshwater fish belonging to the family *Cichlidae*. Though native to Africa, it has now been introduced into many tropical, subtropical and temperate regions of the world (Abdel-Fattah M El-Sayed, 2005). Currently, tilapia is the second most cultured finfish in the world after the carps (Abdel-Fattah M. El-Sayed & Tacon, 1996). In Kenya it accounts for 90% of aquaculture (Charo-Karisa, 2008). Its wide spread culture is due to its fast growth, tolerance to a wide range of environmental conditions, resistance to stress and disease, prolific breeding and easy acceptance of artificial feeds which makes it an ideal candidate for aquaculture (Abdel-Fattah M El-Sayed, 2005; Weaver, et al., 2008). Nutritionally, tilapia is rich in protein. Like other fish species, diet determines its total lipid content and fatty acid composition (Aguiar, et al., 2011; Young, 2009).

Traditionally aquaculture has depended on wild fish harvests for feed ingredients (Pickova, 2009). This is especially true with fish oil which is a rich source of n-3 PUFAs. With the dwindling wild fish harvests coupled with increased demand for fish oil from the neutraceutical food and feed industry (especially for Salmon) (Nichols, Petrie, & Singh, 2010), fish oil prices have gone high making it essential to replace fish oil in

tilapia diets. Tilapia is known to possess the capability to synthesize EPA and DHA from ALA (Karapanagiotidis, et al., 2007); which has allowed inclusion of plant oils like canola and rapeseed in tilapia feeds as a substitute for fish oil. However, with increased interest in biofuels, prices of plant oils are also on the rise (Miller, Nichols, & Carter, 2008). It therefore remains an industry priority to identify new sources of EPA and DHA that do not compete with other human needs for inclusion in tilapia feed. Alternatives being considered include algae and other aquatic plants.

Salvinia molesta is a perennial free-floating aquatic herb belonging to the family *Salviniaceae* (Hasan & Chakrabarti, 2009). Though native to South America it has spread to many tropical and subtropical countries including Kenya (Doeleman, 1989). It grows on slow moving water bodies reproducing vegetatively to form thick mats (Arthur, Stirk, Novak, Hekera, & Staden, 2007) and it doubles every two days. The thick mats prevent sunlight penetration and gaseous exchange, hence cutting off aquatic life below them (IUCN-EARP, 2003). They also cause numerous other problems such as reducing water surface for recreational use, clogging of irrigation systems and creating breeding grounds for mosquitoes and snails (Arthur, et al., 2007; Hasan & Chakrabarti, 2009). To control this weed, various biological, chemical and mechanical methods have been used all with varied levels of success. Tilapia has been tested as one of the possible biological control agents with promising results on fish weight gain (McIntosh, King, & Fitzsimmons, 2003). This has led to interest in *S. molesta*, the nutrients that it may contain and how it can be utilized commercially to make fish feed. A recent study by Dunbar and Deckelbaum (personal communication) revealed that this weed contains both EPA and DHA, in addition to ALA which is common in most plants.

The fatty acid content and composition of tilapia is mainly dependent on its diet. The effect of supplementing commercial tilapia feed with *S. molesta,* which contains some amount of EPA and DHA on the fatty acid profile of tilapia fillet is however unknown. To investigate this, the current study sought to compare the fillet fatty acid profile of tilapia fed on *S. molesta* at various inclusion levels with that fed on commercial control diet only.

1.1. General objective

To determine whether water fern, *Salvinia molesta* can be used as an omega-3 source for cultured tilapia (*Oreochromis niloticus*)

1.2. Specific objectives

1. To determine the proximate composition and omega-3 profile of *S. molesta* and commercial feed used in this study
2. To evaluate the growth response of tilapia (*Oreochromis niloticus*) when fed on diets containing *Salvinia molesta*
3. To determine the omega-3 profile and content of tilapia (*Oreochromis niloticus*) fed on diets containing *Salvinia molesta*

1.3. Justification

Long chain omega-3 fatty acids found in fish are widely recognized to be beneficial for human health and nutrition. They are associated with biochemical and metabolic processes involved in the prevention and reduction of cardiovascular diseases, inflammation and inflammatory diseases as well as some cancers. However the wild catches of fish have been dwindling even as the world human population and indeed the demand for fish continues to increase. This has resulted in a dramatic expansion in aquaculture currently at an annual rate of 9.2% compared to 1.4% for capture fisheries. Even as fish farming is now becoming a major contributor to the world production of food fish, it is important to maintain the high lipid nutritional quality of the product and continue to provide large amounts of the health-promoting n-3 PUFA for the consumer. This can only be done through use of quality feeds as fish do not synthesize omega-3 fatty acids *de novo*. Traditionally aquaculture has relied on fishmeal and fish oil for quality feed but this has become scarce, expensive and unsustainable. It therefore follows that for aquaculture to be sustained there is a need to identify and use cheaper and more sustainable feed ingredients, which on the other hand do not compromise the nutritional value of the fish.

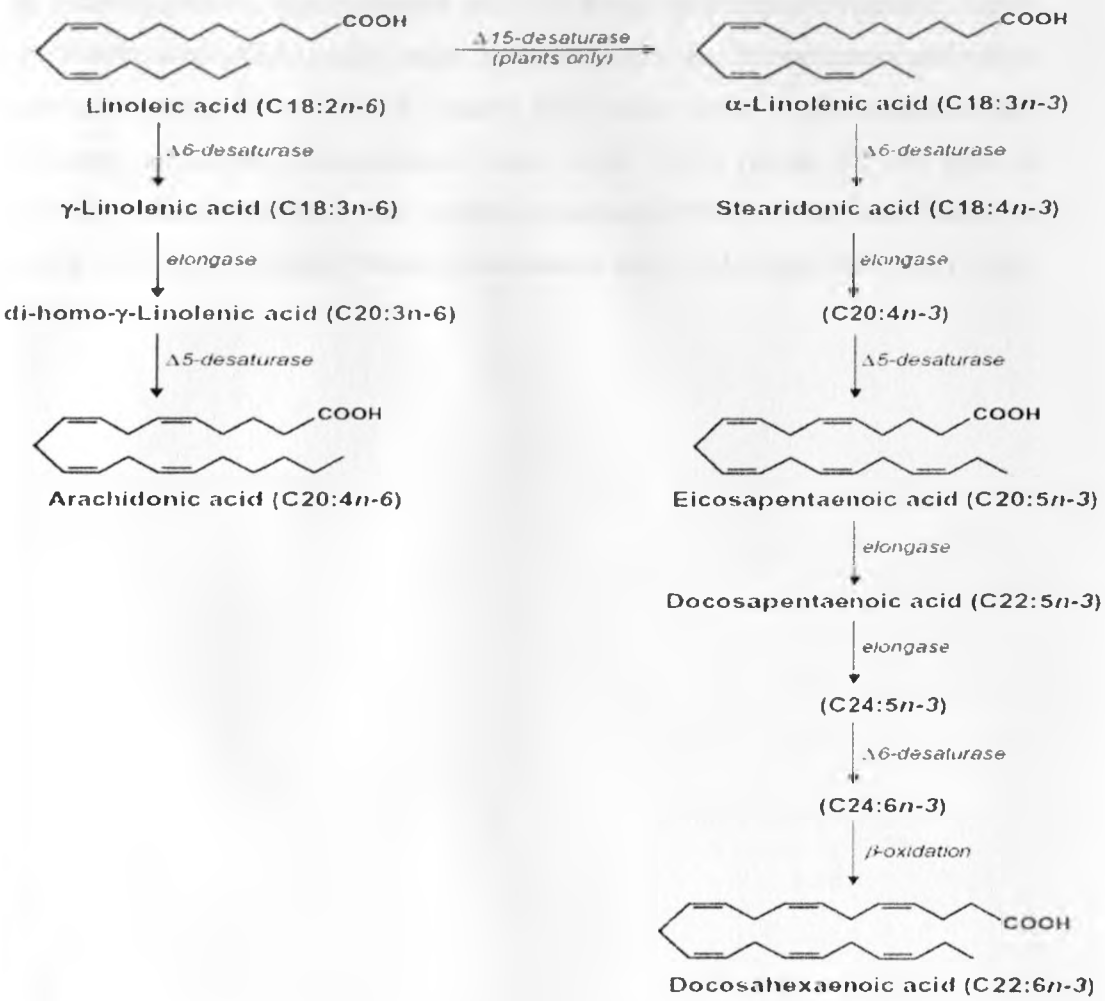
Salvinia molesta an invasive aquatic weed which causes major damage to water systems has been found to contain EPA and DHA though in small amounts. Tilapia has been used as a biological control agent for this weed with promising results. It can therefore be hypothesized that feeding tilapia on *S. molesta* will cause the EPA and DHA to accumulate and hence be available in higher amounts to the human consumer.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1 Essential fatty acids

Essential fatty acids (EFA) refer to fatty acids that are required in the body but cannot be synthesized *de novo* and hence have to be provided through the diet. The essential fatty acids in vertebrates are the 18-Carbon α-linolenic acid (ALA) an omega-3 and linoleic acid (LA) which is an omega-6. In the body, ALA and LA are enzymatically converted to the functionally essential C20 and C22 homologues through a process of elongation and desaturation (figure 1); (Karapanagiotidis, et al., 2007; Roos, et al., 2009). This downstream metabolism of ALA and LA utilizes the same elongases and desaturases and this introduces competition between these two families of fatty acids (Burdge & Calder, 2005). The initial conversion of ALA to 18:4n-3 by the action of Δ^6-desaturase is the rate limiting reaction of the pathway. Though the affinity of Δ^6- desaturase for ALA is greater than for LA, there is greater conversion of n-6 PUFA because there is typically higher concentration of LA than ALA in cellular pools. It therefore follows that conversion of ALA to EPA and subsequently to DHA is limited and humans require a provision of EPA and DHA in their diet (Helene, 1998).



ɟure 1: Biosynthesis pathways of omega-6 and omega-3 polyunsaturated fatty ds (adapted from Roos, et al., 2009)

ŋg chain PUFAs of both the omega-6 and omega-3 families are an important nponent of the cell membrane. On demand, they are liberated from membrane ɔspholipids by the action of various phospholipases to serve as precursors for ɔsanoid metabolism (Larsson, Kumlin, Ingelman-Sundberg, & Wolk, 2004). osanoids are short-lived, hormone-like lipids with a 20 carbon chain. They include kotrienes, prostaglandins and thromboxanes. They play various roles in the body luding; modulation of inflammatory and immune responses and play a critical role in telet aggregation, cellular growth and cell differentiation. Their metabolism is carried

out by cyclooxygenases, lypoxygenases and cytochrome p450 monooxygenases (figure 2). Arachidonic acid (ARA) yields series 2-prostaglandins and thromboxanes and series 4-leucotrienes, while LC-n-3 PUFA mainly EPA yields series 3-prostaglandins and thromboxanes and series 5-leucotrienes (Roos, et al., 2009) (figure 2). The type of eicosanoids produced depends on the relative proportion of PUFAs in cell membranes as well as the cell type. It is mainly through eicosanoids that PUFAs exert their effect in the body.

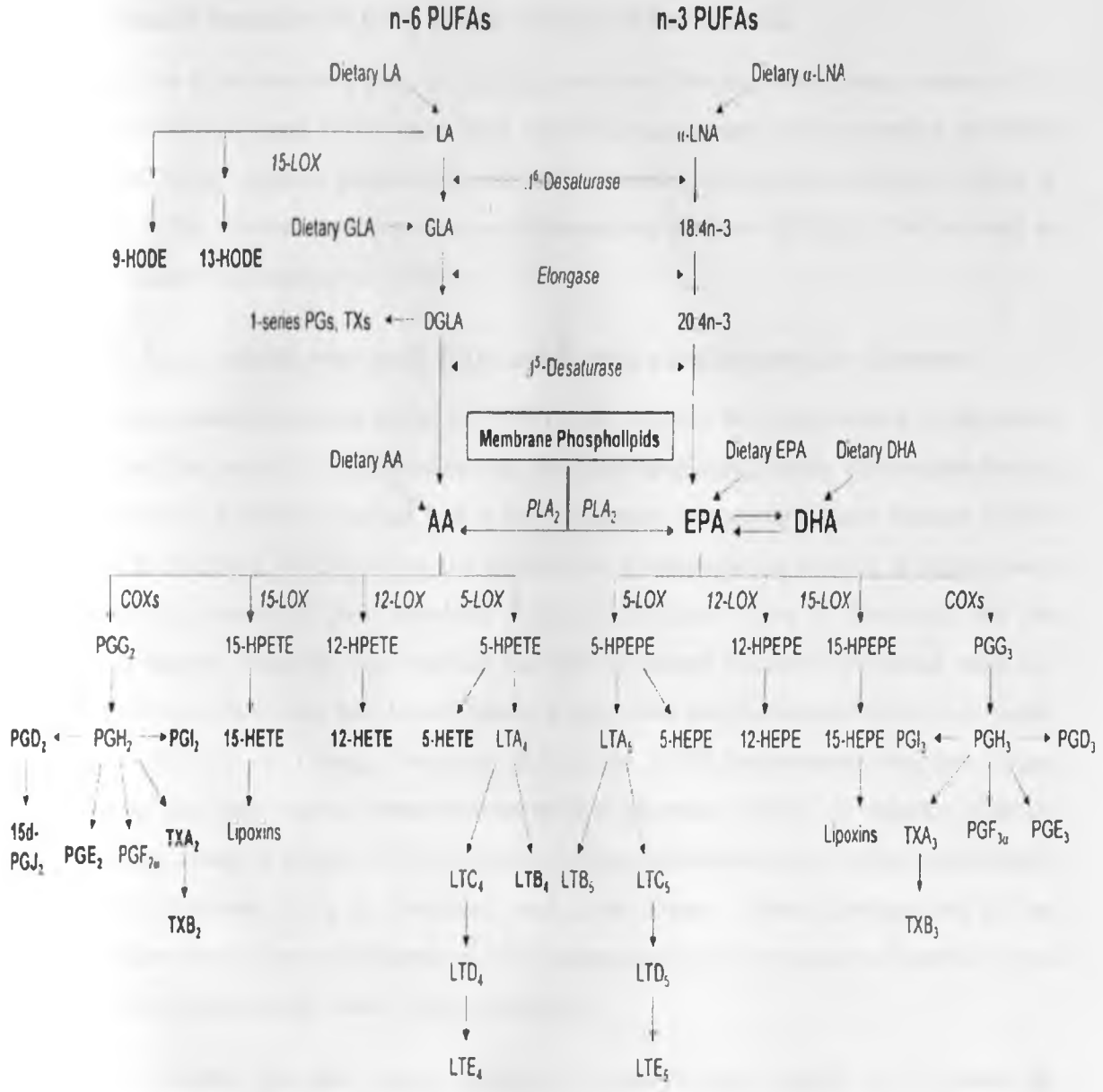


Figure 2: Overview of the metabolism of n-6 and n-3 polyunsaturated fatty acids (PUFAs) into eicosanoids

LA, linoleic acid; LNA, α-linolenic acid; GLA, gamma linolenic acid; DGLA, dihomo-gamma linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid ; PLA2, phospholipase A2; LOX, lipoxygenase; COXs, cyclooxygenases; 15-HETE, 15(*S*)-hydroxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; HEPE, hydroxyeicosapentaenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPEPE, hydroperoxyeicosapentaenoic acid; LT, leukotriene; HODE, hydroxyoctadecadienoic acid; PG, prostaglandin; TX, thromboxane (adapted from Larsson, et al., 2004)

2.2 Health benefits of long chain Omega-3 fatty acids

In addition to the role they play in normal growth and development, dietary intake of LC n-3 PUFAs, EPA and DHA have been positively associated with a number of health benefits. These include prevention and reduction of cardiovascular diseases (Roth & Harris, 2010), inflammation and some inflammatory diseases (Calder, 2006) as well as some cancers (Larsson, et al., 2004).

2.2.1. Long-chain omega-3 fatty acids and cardiovascular diseases

The cardiovascular benefits of LC n-3 PUFAs are perhaps the most studied of the health benefits. They were first observed among the Greenland Inuit people who despite having a diet rich in fat from seafood had a low incidence of coronary heart disease (CHD) (Bang & Dyerberg, 1972). To try and explain this phenomenon a number of studies were carried out comparing the Greenland Eskimos, Eskimos living in Denmark and the Danish people. These studies revealed that the Greenland Eskimos consumed more LC ω-3 PUFAs in their diet, had lower plasma triglyceride and cholesterol levels and higher ratio of EPA to AA (Bang, Dyerberg, & Sinclair, 1980).Furthermore they had longer bleeding time and higher immuno-reactive anti-thrombin AT-III or heparin cofactor (Dyerberg, Bang, & Hjorne, 1975.). Many of these parameters didn't differ significantly between Eskimos living in Denmark and other Danes. These findings led to the conclusion that the lower incidence of CHD among the Eskimos was not of genetic origin but rather of an external factor, presumably diet.

The past decade has also seen a number of clinical trials carried out to prove the cardiovascular benefits of LC n-3 PUFAs. One study carried out among Italian patients surviving myocardial infarction; the GISSI-Prevenzione trial, reported a 15% reduction in death, non-fatal myocardial infarction and stroke among patients treated with EPA+DHA (Gissi Preventione investigators, 1999; Marchioli, et al., 2002). Another study, GISSI-Heart failure, found that EPA and DHA reduced the time to death, and time to death or time to admission for cardiovascular reasons for patients with chronic heart failure (GISSI-HF investigators, 2008)

Amid the increasing evidence of the cardiovascular and other benefits of LC ω-3 PUFAs, their mechanism of action remains unclear. A few proposals have however been put forward (Seo, Blaner, & Deckelbaum, 2005; Sudhendran, Chang, & J, 2010);

a) **Anti-inflammatory effect**: Inflammation is recognized to play a central role in the development and progression of coronary artery disease (CAD). This process is driven by the inflammatory eicosanoids yielded from omega-6 (ω-6) metabolism. Omega-3 PUFAs reduce inflammation through enzymatic competition in the synthesis of eicosanoids (Din, Newby, & Flapan, 2004); antagonizing the effect of ω-6 eicosanoids; reducing expression of cell adhesion molecules and suppressing pro-inflammatory cytokines (Calder, 2006; DeCaterina, Liao, & Libby, 2000).

b) **Improved endothelial function:** Individuals with cardiovascular risk factors or established coronary heart disease have abnormal endothelial function. Omega-3 PUFAs, especially DHA, have been found to improve endothelial vasomotor function through improving vasodilation and improving systemic arterial compliance (Stebbins, Stice, Hart, Mbai, & Knowlton, 2008).

c) **Blood pressure:** through the effect on endothelial function, ω-3 PUFAs lower blood pressure. However high doses of fish oil (about 3.6g/day) are required (Cicero, Ertek, & Borghi, 2009).

d) **Triglyceride lowering:** ω-3 PUFAs lower triglyceride concentrations by lowering the assembly and secretion of very low density lipoproteins (VLDL) (Davidson, 2006). This reduction is dose dependent. A reduction of 25-30% in serum triglycerides has been observed with doses of 4g/day (Din, et al., 2004).

e) **Atherosclerosis:** Animal and human studies have revealed that ω-3 PUFAs protect against progression of atherosclerotic plaques. These effects could be due to reduction in lipids, inflammation, production of growth factors; and suppression of smooth muscle cell proliferation (Din, et al., 2004; Patterson, Wall, Fitzgerald, Ross, & Stanton, 2012).

f) **Thrombosis:** Long-chain ω-3 PUFA decrease the risk of atherothrombosis by affecting platelet aggregation. This is through competitive incorporation of EPA and DHA into the platelet phospholipids at the expense of the ω-6 PUFAs. Competitive synthesis of eicosanoids also inhibits production of the pro-aggregatory thromboxane A_2 originating from arachidonic acid (DeFilippis, Blaha, & Jacobson, 2010; Engstrom, Wallin, & Saldeen, 2001).

g) **Arrhythmias:** In the GISSI- prevenzione trial, LC ω-3 PUFA were found to decrease sudden death through prohibiting cardiac arrhythmia (Gissi Preventione investigators, 1999).

2.2.2. Long chain ω-3 PUFAs and Cancer

Long chain Omega-3 FAs have been shown to decrease the relative risk of breast, prostate and colon cancers. A prospective study involving 47,882 men for a period of 12 years found that eating fish more than three times per week was associated with a reduced risk of prostate cancer (Augustsson, et al., 2003).

Studies with human cancer cell lines, animal models and preliminary trials with human subjects have also shown that administration of EPA and/or DHA alters the toxicities and activity of chemotherapeutic drugs (Biondo, Brindley, Sawyer, & Field, 2008).

2.2.3. Long chain ω-3 PUFAs, inflammation and inflammatory diseases

Inflammation is a normal physiological process in response to infection or injury (Calder, 2006). It serves to begin the process of eliminating the invading pathogens and toxins and repair of damaged tissues. However excessive and inappropriate inflammation which is characterized by overproduction of inflammatory cytokines and eicosanoids is harmful. It contributes to a range of acute and chronic human diseases as well as autoimmune diseases.

Sufficient intake of long chain omega-3 PUFAs decreases inflammation. This is believed to act through

which counters the effect of methylmercury (Park & Mozaffarian, 2010). Though such pollutants should be a concern to large fish which are long-lived and higher in the marine food chain (Mozaffarian & Rimm, 2006) the public often tends to generalize marine fish and this becomes a cause of alarm.

There is also a question of sustainability of marine fish as a source of LC ω-3 PUFAs (Racine & Deckelbaum, 2007). The world population has continued to rise reaching 7 billion in late 2011. The populace has also become more aware of the health benefits of LC ω-3 PUFAs. These two factors have caused an increase in the intake of fish. However, wild fish captures are dwindling and it is clear they will not meet the growing fish demand (Weaver, et al., 2008). This has led to the increase in aquaculture as a possible way of increasing world fish production and meet consumer need. Globally, aquaculture has been growing at the rate of 8.3% per annum. This has seen its contribution to total fish production rise from 3.9% in 1970 to 36.9% of 2008 (FAO, 2010).

2.4. Aquaculture in Kenya

For a long time, Kenya has depended on capture fisheries to meet most of its fish requirement. However, with increasing awareness of the health benefits of fish consumption, dwindling capture fisheries and increased population growth across the country, aquaculture is now considered a serious alternative (Charo-Karisa, 2008). This is especially so because production in the Kenyan portion of Lake Victoria, the single most important fishery are on the decline (Charo-Karisa, 2008). The rest of the productive sources like Lake Naivasha, Lake Turkana, Lake Baringo and smaller Lakes like Chala and Jipe are equally at advanced levels of exploitation.

Aquaculture in Kenya can be categorized into three broad groups, namely warm fresh water aquaculture, cold fresh water aquaculture and mariculture. Warm fresh water aquaculture is the most developed of the three and involves production of various species of Tilapia and African catfish (*Clarias gariepinus*) under semi-intensive systems (Mwangi, 2008). Culture of the various tilapia species constitutes 90% of aquaculture in

Kenya. Polyculture of tilapia and African catfish is also practiced mainly to control the prolific breeding of the former.

The government of Kenya recognizes that aquaculture would not only increase fish availability and decrease pressure on the natural fisheries but also alleviate poverty in the rural populations. It is for this reason that in the Economic Stimulus Programme (ESP), the government set aside Ksh. 3 billion ($37.5 million) for pond construction and stocking.

2.5. Aquaculture as an omega-3 source

In the wild, fish obtain LC n-3 PUFAs from their diet of marine microalgae and small aquatic plants (Nichols, et al., 2010). Equally, farmed fish do not naturally synthesize LC n-3 PUFAs and instead depend on what is supplied in their diet. Research has shown that the lipid content and fatty acid profile of fish is dependent on diet (Grisdale-Helland & Helland, 1997; Hardy & Lee, 2010; Jobling & Johansen, 2003). In the US for example corn forms a large portion of feed for cultured tilapia. This yields fish with a potentially harmful fatty acid combination (high ARA: EPA) making it no different from a hamburger (Wake Forest University Baptist Medical Center, 2008; Weaver, et al., 2008).

To provide for LC n-3 PUFAs in tilapia diets, fish oil is used as a major lipid source in tilapia feed. It is also used to coat extruded pellets to improve palatability and appearance of the feed. Fish oil also aids in the absorption of fat soluble vitamins, provides precursors of hormones and prostaglandins and also building blocks of cellular and membrane structures. There has however been stagnation in global fish oil production as well as an increase in demand for it for use in salmon feeds which has caused inflation in fish oil prices (Alder, Campbell, Karpouzi, Kaschner, & Pauly, 2008). It has therefore become an industry priority to replace fish oil with cheaper and more sustainable sources of dietary lipid in tilapia feeds. Before fish oil can be replaced in tilapia diets it is however important that we understand the dietary requirements of tilapia.

Research has shown that replacement of fish oil with vegetable oils in tilapia diets does not affect growth. However it influences the fillet fatty acid profile as this is markedly

influenced by dietary fatty acid composition. Use of vegetable oils which lack EPA and DHA in tilapia feeds decreases the concentration of the beneficial LC n-3 PUFAs in fish fillet destined for the human consumer (Karapanagiotidis, et al., 2007). A solution to this is the use of finishing diets with fish oil during the last stage of grow-out just before harvesting. Ng and Chong (2004) however found out that after 3 months of wash out, fillet of tilapia fed 100% crude palm oil or soybean oil had significantly lower levels of EPA and DHA when compared to those fed fish oil throughout grow-out (Ng & Chong, 2004).

Some vegetable oils such as soybean oil however contain high levels of linoleic acid (an omega-6), and high levels are deposited in the tilapia fillet and significant levels are observed even after wash-out with fish oil. Modern foods already contain too much omega-6 PUFAs and a good fish oil substitute should limit the deposition of n-6 in the fish fillet. Another challenge to using vegetable oils is their increased use in biofuels which has caused their demand and price to go up (Miller, et al., 2008). Besides, the conversion of ALA found in vegetable oils to the EPA and DHA is insufficient to meet the requirement by tilapia and hence dietary supplementation of preformed PUFAs is still necessary.

2.6. *Salvinia molesta*

Salvinia molesta commonly known as water fern or giant Salvinia is a free floating clonal fern that reproduces only vegetatively (Hasan & Chakrabarti, 2009). The plant is native to Brazil but has now found its way to other parts of the world (McIntosh, et al., 2003) including Kenya (IUCN-EARP, 2003). Though it can survive a wide range of temperatures (-3°C to 43°C) it achieves optimal growth at temperatures between 24-28°C (Cary & J, 1983). With adequate nutrients it doubles every 2.2 days forming thick mats covering the water surface (Hasan & Chakrabarti, 2009)

At low populations, *Salvinia* has favorable effects especially on fisheries acting as a refuge for fry and as a source of food organisms (IUCN-EARP, 2003). However, the thick and extensive mats it forms are quite harmful. The mats prevent light penetration, decrease gaseous exchange and increase biological oxygen demand thus cutting off

aquatic life beneath them (Hasan & Chakrabarti, 2009). The mats also reduce water surface for recreational activity; block irrigation canals; make fishing difficult and form breeding places for disease vectors (McIntosh, et al., 2003).

Various methods have been used to control the weed. These include the use of herbicides such as paraquat, biological control using the *Cyrtobagous salviniae* weevils and mechanical means using hand pulled nets. All these methods have successfully brought the weed under control but not without some challenges. Large masses of dead decaying matter after spraying for example, reduced the dissolved oxygen (IUCN-EARP, 2003). Current research has however shifted into looking at means to better utilize the weed. McIntosh and others (2003) evaluated its utility as a feed for tilapia. Good results were obtained when *S. molesta* was combined with commercial feed. They however concluded that since the weed has a high water content in its fresh state, drying it may yield better results (McIntosh, et al., 2003). Dunbar and Deckelbaum (Personal communication) also found that this aquatic plant contains significant amounts of ALA, EPA and DHA. This suggests that this plant may be a good source of the much needed EPA and DHA for inclusion in tilapia feeds.

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Feed preparation

Salvinia molesta was collected from a pond in Karen (Nairobi, Kenya) and dried under a shade for two days. This was then ground using a kitchen blender and sifted through a tea strainer to get particles of about 1mm. The commercial feed used in this study was purchased from Crop King Company (Nakuru, Kenya).This particular feed was selected for the study because the supplier had been contracted by the government to supply tilapia farmers. To prepare the experimental diets, the commercial feed and *Salvinia molesta* were weighed and mixed in the ratio of 10:0, 9:1, 8:2 and 7:3 (commercial feed: *Salvinia molesta*). These ratios were arrived upon after taking into consideration that farmed tilapia require at least 50% of the protein in their diet be of animal origin.

3.2. Determination of the proximate composition of experimental diets

The composition of the commercial feed and *Salvinia molesta* was evaluated. The proximate composition of the experimental diets was then mathematically derived.

3.2.1 Determination of crude protein

Crude protein was determined by the Kjeldal method. Briefly, 0.2g of the sample was weighed in duplicate on a piece of nitrogen free, ashless whatman filter paper. The paper was folded and put in a kjeldal flask into which a kjeldahl catalyst tablet and 20ml of concentrated sulphuric acid were added. This was then heated on a block digester in a fume cupboard until a clear solution was obtained (about 3hrs). After cooling, distilled water was added to the kjeldahl flask to about ¾ full and drops of phenolphthalein indicator added.

A 400ml conical flask with 25ml of 0.1N hydrochloric acid and drops of methyl orange indicator was placed in the outlet of the distillation unit. The kjeldal flask was then connected to the distillation unit and 40% NaOH solution added till the colour changed.

Distillation was then allowed to take place until the volume in the conical flask rose to 200ml. The distillate was then back titrated with 0.1N sodium hydroxide until the colour changed to yellow.

Crude protein content was calculated using the formula:

$$Crude\ protein = \frac{Vol \times N \times 10^{-3} \times 14.0067 \times 100}{total\ weight\ of\ sample} \times 6.25$$

Vol- Volume of NaOH used

N- Normality of NaOH

3.2.2. Determination of Lipid content

To determine the lipid content of the experimental diets, lipids were extracted using modified Folch method (Folch, Lees, & Stanley, 1956). Briefly, 10g of feed sample was weighed into a conical flask. 50ml of chloroform/methanol (2:1) was added and the mixture transferred onto an orbital shaker (2 hrs). This was then followed by filtration through glass wool and transfer of the filtrate into a separating funnel. The residue was recovered and lipids re-extracted with 50ml of chloroform/methanol (2:1) for another one hour on an orbital shaker followed by filtration. The filtrate was washed by addition of 0.2 volumes of 0.5% sodium chloride, mixed well and left to stand (1hr) for phase separation. The lower phase (chloroform phase) was recovered and placed in a dry, pre-weighed volumetric flask. Chloroform was then evaporated in an oven at 55°C. The weight of the lipids was obtained by subtracting the weight of the empty flask from the weight of flask with lipids.

The same procedure was carried out for *Salvinia molesta* but using 4g of sample. This is because of the voluminous nature of its dried ground state.

The percentage lipid content was determined as;

$$Lipid\ content\ \% = \frac{weight\ of\ lipid\ in\ grams}{weight\ of\ sample\ in\ grams} \times 100$$

3.2.3. Determination of lipid profile

To determine the lipid composition of the commercial feed and *Salvinia molesta*, a thin layer chromatography (TLC) was carried out. Briefly, lipid samples were diluted in extraction solvent (chloroform: methanol- 2:1). The TLC tank was equilibrated with developing solvent (Hexane-Diethyl ether-Acetic acid ratio of 50:50:1) for 1 hour. Lipids were then spotted on a TLC plate using a capillary tube and developed in the TLC tank and visualized using iodine. Lipid classes in each sample were identified by using the retardation factor (RF) values against standards.

3.2.4 Determination of fatty acid profile

3.2.4.1. Preparation of FAME

Fatty Acid Methyl Esters (FAME) were prepared by acid catalyzed transesterification following the procedure described by Mbatia *et. al.* (2010). Briefly, 20 mg of lipid sample was mixed with 2ml of toluene, followed by addition 2ml of 1.5% of Sulphuric acid in dry methanol. After mixing well, the mixture was incubated at 55°C overnight. 4 ml of saturated NaCl solution was added vortexed and 2 ml of hexane (HPLC grade) added. To the mixture 3ml of sodium bicarbonate (2% of $NaHCO_3$) was added and mixed well. The mixture was then allowed to separate into an upper and lower phase. 180 µl of the upper phase was taken for gas chromatography (GC) analysis. Before analyzing this on a GC machine an internal standard (C 13:0) was added to have a final concentration of 0.5mM. A thin layer chromatography was run again to confirm that derivatization had occurred before GC analysis.

3.2.4.2 Gas chromatography

Fatty acid methyl esters (FAMEs) were separated and quantified using a gas chromatography (Varian chrompack CP 3800 GC) system equipped with a flame ionization detector (FID). Supelcowax^{TM} 10 fused silica capillary column (60 m x 0.32 mm x 0.25 µm film thickness; Supelco, Bellefonte, PA, USA) was used to separate FAME. The carrier gas was helium at 550kpa. The temperature programme for separation was as follows:

- Initial column oven temperature of 35°C held for 3 min.
- Column oven temperature increased to 240 at 10°C/min and held for 35 min.
- The detector temperature was kept constant at 300°C.

The instrument was calibrated with a one-point calibration method, using a standard mixture of fish oil FAME of known proportions (Qualmix fish S). The response factors of the different methyl esters were obtained from analysis of the standard mixture and were used to calculate the relative amounts of different fatty acids in a sample based on mol%. These data were compared with the internal standard (methyl tridecanoate) to determine the absolute amount (mM) of the fatty acids in the sample. GC data provided are based on duplicate measurements.

3.2.5. Determination of ash content

To determine the ash content in the commercial feed and the *Salvinia molesta*, 3g of sample was weighed into a crucible and heated in a muffle furnace at 550°C overnight. The residue was weighed and ash content determined as;

$$Ash\ content\ (\%) = \frac{weight\ of\ residue}{weight\ of\ sample} \times 100$$

3.2.6. Determination of crude fiber

Crude fiber was quantified by weighing 3g of sample and digesting it in 1.8% H_2SO_4 for 30min. The remaining sample was rinsed in hot water then digested in 1.8% KOH for another 30min. There-after the sample was rinsed three times in hot water, dried in an oven at 60°C overnight, cooled in a desiccator and weighed (initial weight). It was then heated in a muffle furnace at 550°C for 4h cooled in a desiccator and weighed again (final weight). The fiber content was calculated as;

$$Crude\ fiber = \frac{initial\ weight - final\ weight}{Sample\ weight} \times 100$$

3.2.7. Determination of Nitrogen free extract

The nitrogen free extract (NFE) was determined using the formula

$$NFE = dry\ matter - crude\ protein - crude\ fiber - lipid\ content - Ash$$

3.3. Feeding Trial

Monosex male tilapia fingerlings weighing 0.35 ± 0.03g (mean ± S.E.) were obtained from Omega Farm on Olkokwa Island, Lake Baringo. From this, 50 fish were randomly picked to serve as the baseline sample and the rest were weighed in groups of 100 and each group assigned to a labeled hapa suspended on a water filled concrete fish pond. In triplicate they were fed on the experimental or control diets (Table 1) for a period of 56 days. The fish were fed four times daily at the rate of 10% body weight/day for the first two weeks, and 5% bodyweight per day thereafter. The fish were weighed every two weeks and the feed ration determined.

Table 1: Composition of experimental diets

Treatment Group	Commercial feed (%)	*Salvinia molesta* (%)
Control	100	0
10% *S. molesta*	90	10
20% *S. molesta*	80	20
30% *S. molesta*	70	30

Experimental diets contained *S. molesta* at various inclusion levels ranging from 0% to 30%

A sample of 30 fish was taken from each hapa at the end of the experimental period and their fillet taken for laboratory analysis. Fillet obtained from fish from the same cage was treated as one pooled sample. The muscle was preserved frozen at 4°C prior to the laboratory analysis.

3.4. Evaluation of fish performance

To determine growth response of the fish to the experimental diets the following parameters were monitored as follows:

- $Mean\ weight\ gain(MWG) = final\ mean\ weight - initial\ mean\ weight$
- $Feed\ conversion\ ratio(FCR) = weight\ of\ feed\ given(g)\ /fish\ weight\ gain(g)$
- $Relative\ Growth\ Rate\ (RGR)\% = \left(\frac{final\ average\ weight - initial\ average\ weight}{initial\ weight}\right) \times 100$
- $Protein\ Efficiency\ Ratio\ (PER) = fish\ weight\ (g)\ /\ (protein\ intake\ (g)$
- $Specific\ growth\ rate\ (SGR)\% = \left(\frac{\log final\ weight - \log initial\ weight}{time\ in\ days}\right) \times 100$
- $Mortality\ rate = \frac{Number\ of\ deaths}{total\ number\ of\ fish} \times 100$
- $Increase\ in\ length = final\ mean\ length(mm) - initial\ mean\ length(mm)$

3.5. Water quality analysis

Water quality is known to affect feeding, growth and survival rate of fish. To ensure that water parameters remain within the optimum for fish performance, temperature and dissolved oxygen were measured daily, in the morning (0600hrs) and afternoon (1500hrs). Both parameters were measured using DO & temperature meter (Hanna instruments model H1 9146).

3.6. Lipid extraction from fish muscle and fatty acid profile analysis

For examination of the lipid content and fatty acid composition from the edible muscle of fish, the same procedure as that carried on the feed was carried out (section 3.2.2). For

lipid extraction however 2g of lyophilized fish muscle and 20ml of extraction solvent (chloroform/methanol 2:1) was used. The rest of the procedure was the same as with feed sample (section 3.2.2).To determine the lipid and fatty acid profiles for the fish oil the same procedure of thin layer chromatography, fatty acid methyl ester (FAME) preparation and gas chromatography was carried out as for feeds (sections 3.2.3 and 3.2.4).

3.7. Data analysis

Data from each cage was treated as independent sample forming three samples for each treatment. Proximate composition of the diets, growth performance and feed utilization efficiency parameters, muscle total lipid and fatty acid composition were all subjected to one-way analysis of variance (ANOVA) and differences were considered significant at $p \leq 0.05$. Analyses were performed using SPSS statistical package (version 16, SPSS Inc., Chicago, USA).

CHAPTER FOUR

4.0. RESULTS

4.1. Proximate composition of commercial feed, *S. molesta* and experimental diets

Proximate analysis of the feeds revealed that the test plant *Salvinia molesta* has high ash content (31.63%) while the commercial feed was mainly rich in the nitrogen free extract (Table 2). The commercial feed contained 14.95% protein in contrast to the 37% protein indicated on the manufacturer's label.

Table 2: Proximate composition of commercial feed and test plant *Salvinia molesta*

Feed component	Commercial feed	*Salvinia molesta*
Crude Protein %	14.95±0.86	18.38±.00
Total Lipid %	3.90±0.1	3.00±0.1
Crude Fiber %	12.07±0.1	18.30±.37
Ash %	6.93±0.03	31.63±.24
Moisture %	9.96±1.8	7.14±.00
NFE %	52.20±.94	21.55±.03

Values represent mean ± SE

Results on this table represent the average of two samples of commercial feed and *S. molesta*. NFE= nitrogen free extract.

The composition of the experimental diets was mathematically derived from Table 2 above. Protein content in the experimental diets increased with increase in amount of *S. molesta* (Table 3). The diet with the highest inclusion of *S. molesta* (30% *S. molesta*) had the highest content of protein. However there was no significant difference in the protein content between the three experimental diets and the control. The total lipid content and moisture were highest in the control diet. However this did not differ significantly from the experimental diets. In contrast ash content, crude fiber and NFE were significantly different across groups (Table 3).

Table 3: Proximate composition of experimental diets

	Experimental diet			
Feed component	Commercial feed (control)	10% *S. molesta*	20% *S. molesta*	30% *S. molesta*
Crude Protein %	14.95±.96[a]	15.29±.77[a]	15.63±.68[a]	15.97±.60[a]
Total Lipid %	3.90±.10[a]	3.81±.10[a]	3.72±.10[a]	3.63±.10[a]
Crude Fiber %	12.07±.10[a]	12.69±.13[ab]	13.32±.15[bc]	13.94±.18[c]
Ash %	6.93±.03[a]	9.40±.05[b]	11.87±.07[c]	14.34±.09[d]
Moisture %	9.95±1.83[a]	9.67±1.64[a]	9.39±1.46[a]	9.11±1.30[a]
NFE %	52.20±.94[a]	49.14±.85[ab]	46.07±.76[bc]	43.01±.67[c]

Values represent the mean ±SE. NFE= nitrogen free extract

Figures in the same row that have the same superscript letter do not differ significantly ($p \leq 0.05$).

There was so significant difference in crude fiber and NFE content between the following groups: control and the 10% *S. molesta* groups, 10% *S. molesta* and 20% *S. molesta* groups and 20% *S. molesta* and 30% *S. molesta* groups. However the two parameters differed significantly between the control and diets containing *S. molesta*

4.2 Fatty acid composition of commercial feed, *S. molesta* and experimental diets

Linoleic acid (18C omega-6) was the most abundant fatty acid in the commercial feed 19.67 ±.37mg/g of feed (Table 4). No arachidonic acid was detected in commercial feed. However ALA, EPA, DPA and DHA were present in the commercial feed albeit in small amounts. *S. molesta* was found to be rich in hexadecanoic acid (16C SFA, 34.93%). The ratio of LA to ALA in *S. molesta* was 0.8:1. In contrast the ratio of LA to ALA in the control diet was 42:1. The ratio of omega-6 to omega-3 in the commercial feed was 32:1 in contrast to 0.6:1 in *S. molesta* (Table 4).

Table 4: Fatty acid composition of Commercial feed and *Salvinia molesta*

	Commercial feed	*Salvinia molesta*
SFA	**Mean± S.E.**	**Mean± S.E.**
Tetradecanoate (C14:0)	0.17±.01	5.26±2.57
Pentadecanoate (C15:0)	0.20±.03	1.42±1.24
Hexadecanoate (C16:0)	8.98±.98	10.36±1.88
Stearate (C18:0)	0.27±.09	0.91±.20
Total SFA	**9.63±.87**	**17.94±1.73**
MUFAs		
Palmitoloate (C16:1)	0.17±.02	3.03±1.12
Oleate (C18:1)	8.59±.95	2.31±.05
Vaccenate (C18:1)	ND	0.99±.52
Eicosenoate (C20:1)	0.11±.05	0.15±.07
Erucate (C22:1)	0.05±.02	0.43±.31
Total MUFAs	**8.93±1.0**	**6.89±1.35**
n-6 PUFAs		
Linoleate (C18:2)	19.67±.37	1.66±1.28
Arachdidonate (C20:4)	ND	0.31±.13
Docosatetraenoate (C22:4)	0.09±.01	0.03±.03
Total n-6 PUFAs	**19.75±.37**	**2.00±1.38**
n-3 PUFAs		
Linolenate (C18:3)	0.47±.19	2.06±1.04
Octadecatetraenoate (C18:4)	0.02±.02	0.13±.02
Eicosapentaenoate (C20:5)	0.10±.06	0.25±.12
Docosapentaenoate (C22:5)	0.08±.01	0.64±.23
Docosahexaenoate (C22:6)	0.03±.01	0.11±.01
Total n-3 PUFAs	**0.71±.24**	**3.18±.1.41**
Total FA	**39.00**	**30.00**
n-6:n-3	**32:1**	**0.56:1**

Values here represent the weight of individual fatty acids in a gram of either commercial feed or *Salvinia molesta.*

There was no significant difference in the content of FA between the commercial feed and *S. molesta* except for Oleate, Linoleate, total n-6 PUFAs and Docosahexaenoate. ($p \leq 0.05$)

ND = not detectable (levels below 0.01mg/g of sample feed)

Omega-6 fatty acids formed the largest portion of the commercial feed (50.81%) while saturated fatty acids formed the largest portion of the *S. molesta* (Fig. 3). The level of omega-6 fatty acids was about 10 times more in the commercial diet than in *S. molesta*. In contrast there was a higher (4.5 times more) content of omega-3 FAs in *S. molesta* than in the commercial diet.

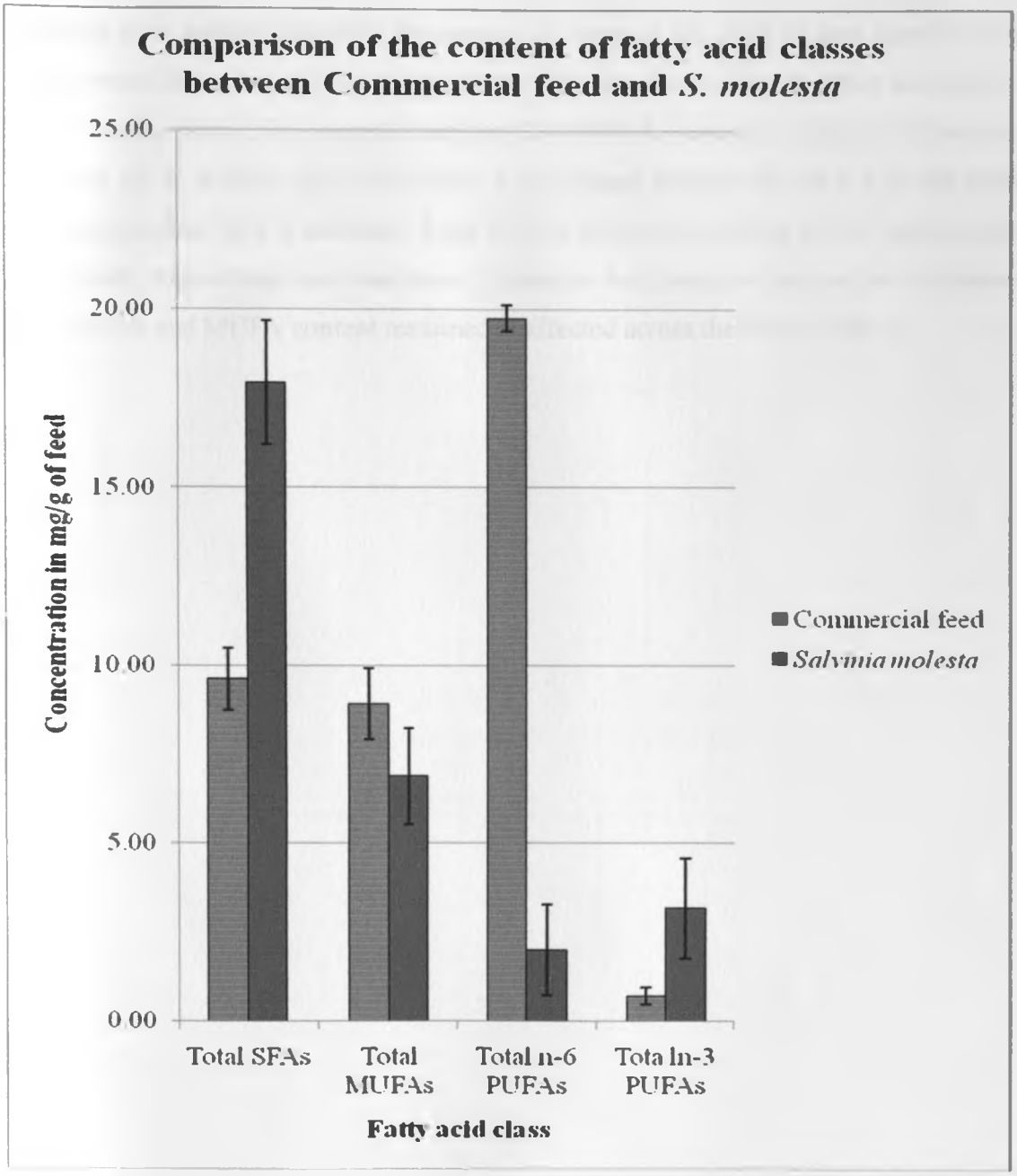


Figure 3: Comparison of the fatty acid class content between commercial feed and *S. molesta*

Inclusion of *S. molesta* increased the amount of omega-3 per gram of feed from 0.71mg in the control to1.45mg in the diet containing 30% *S. molesta*. This in effect reduced the n-6: n-3 ratio from 32:1 (control diet) to 22:1(30% *S. molesta*) (Table 5). However inclusion of *S. molesta* also introduced a significant amount of ARA into the diets (0.09mg/g of diet, 30% *S. molesta*). Total SFA in the diet containing 30% *S. molesta* was significantly higher than the other diets. In contrast total omega-6 reduced with increase in *S. molesta* and MUFA content remained unaffected across the diets (Table 5).

Table 5: Fatty acid composition of the various experimental diets (mg/g of diet)

	Commercial feed (Control)	10% *S. molesta*	20% *S. molesta*	30% *S. molesta*
SFA	**Mean± S.E.**	**Mean± S.E.**	**Mean± S.E.**	**Mean± S.E.**
Tetradecanoate	0.17±.01	0.68±.15	1.19±.30	1.70±.44
Pentadecanoate	0.20±.03	0.32±.07	0.44±.14	0.57±.21
Hexadecanoate	8.98±.98	9.12±.52	9.26±.50	9.39±.51
Stearate	0.27±.09	0.34±.05	0.40±.05	0.46±.05
Total SFA	**9.63±.87** [a]	**10.46±.46** [a]	**11.29±.45** [a]	**12.12±.46** [b]
MUFAs				
Palmitoloate	0.17±.02 [a]	0.46±.07 [a]	0.74±.13 [ab]	1.03±.19 [b]
Oleate	8.59±.95	7.96±.49	7.34±.44	6.71±.38
Vaccenate	ND	0.10±.03	0.20±.06	0.30±.09
Eicosenoate	0.11±.05	0.11±.03	0.11±.02	0.12±.02
Erucate	0.05±.02	0.09±.02	0.13±.04	0.17±.05
Total MUFAs	**8.93±1.0**	**8.72±.52**	**8.52±.49**	**8.31±.46**
n-6 PUFAs				
Linoleate	19.67±.37 [a]	17.86±.21 [b]	16.06±.23 [c]	14.26±.27 [d]
Arachidonate	ND [a]	0.03±.01 [a]	0.06±.01 [ab]	0.09±.02 [b]
Docosatetraenoate	0.09±.01	0.08±.00	0.07±.00	0.07±.01
Total n-6 PUFAs	**19.75±.37** [a]	**17.97±.20** [b]	**16.20±.23** [c]	**14.42±.28** [d]
n-3 PUFAs				
Linolenate	0.47±.19	0.63±.11	0.79±.15	0.95±.19
Octadecatetraenoate	0.02±.02	0.03±.01	0.04±.01	0.05±.01
Eicosapentaenoate	0.10±.06	0.12±.03	0.13±.03	0.15±.03
Docosapentaenoate	0.08±.01	0.14±.03	0.19±.06	0.25±.09
Docosahexaenoate	0.03±.01 [a]	0.04±.00 [ab]	0.04±.00 [ab]	0.05±.00 [b]
Total n-3 PUFAs	**0.71±.24**	**0.95±.16**	**1.20±.22**	**1.45±.31**
Total FA	**39.00** [a]	**38.10** [b]	**37.20** [c]	**36.30** [d]
n6:n3	**32:1**	**29:1**	**25:1**	**22:1**

Values represent weight of FA in mg/g of diet

Rows with superscript letters indicate values that were statistically different across groups. The rest had no significant difference ($p \leq 0.05$). ND = not detectable (value<0.01mg/g of diet)

4.2. Growth response of the fish

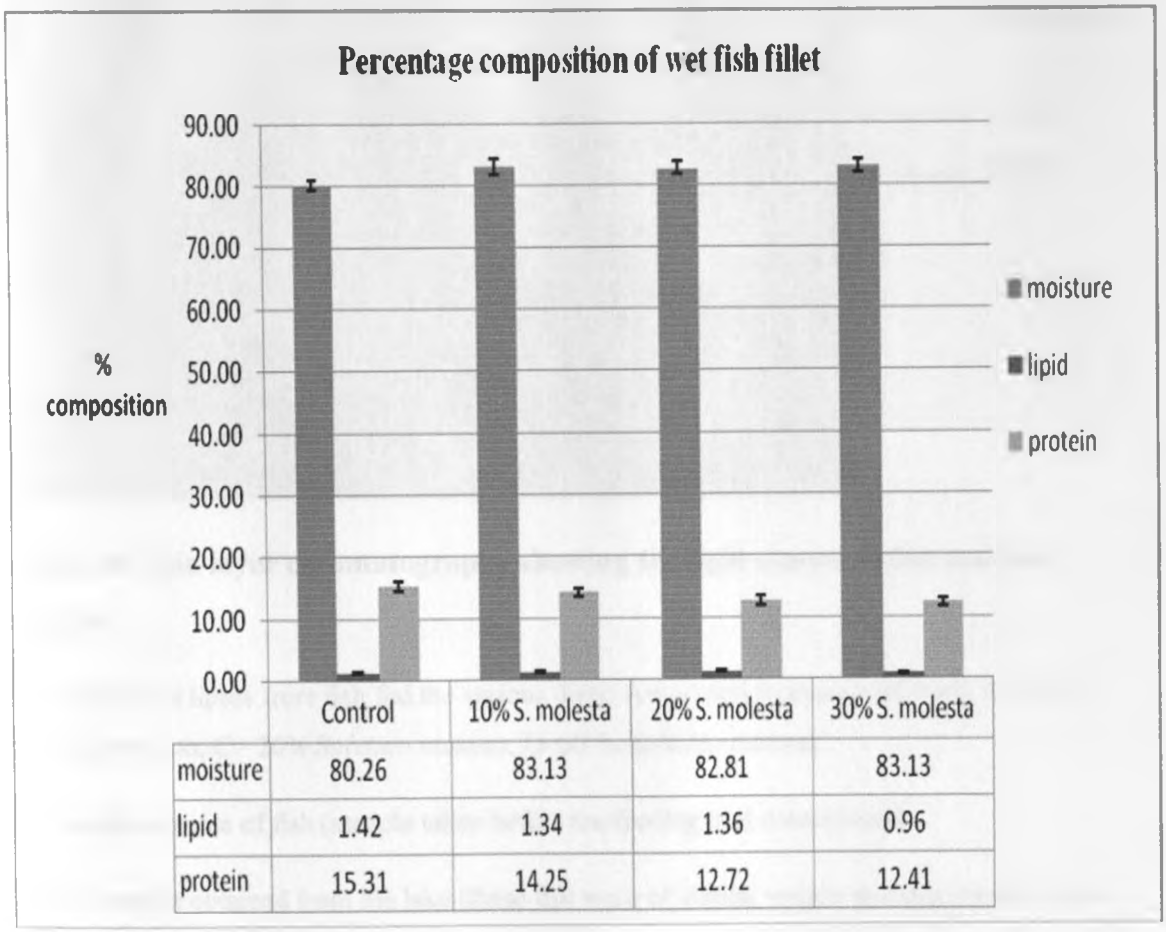
Water temperature and dissolved oxygen were monitored twice daily during the experimental period and were found to be within acceptable levels for fish growth (22-29°C and >3mg/l). There was no significant difference observed in the two parameters between cages or tanks.

Fish receiving the commercial control diet recorded the highest weight gain as well as increase in length. Increase in both weight and length tended to decrease with increase in *S. molesta* in the diet, however fish receiving the diet containing 30% *S. molesta* performed better than those receiving 20% *S. molesta.*

Fish receiving the commercial control diet had the highest PER and this decreased with increase in *S. molesta* in the diet. Mortality was highest in the fish receiving 20% *S. molesta.* For all the growth parameters however, there was no significant difference in performance across the groups (Table 6)

4.3. Effect of diet on the moisture, lipid and protein composition in fish fillet

A large portion of the fillet from all the dietary treatment groups was composed of water (80-83%). Protein content was in the range of 12-15% while lipid content ranged between 0.96-1.43 %.



	Control	10% S. molesta	20% S. molesta	30% S. molesta
moisture	80.26	83.13	82.81	83.13
lipid	1.42	1.34	1.36	0.96
protein	15.31	14.25	12.72	12.41

Figure 4: Percentage content of moisture, lipids and proteins in the wet fish fillet fed on diets with various inclusion levels of *S. molesta*

A thin layer chromatography to separate the lipids from fish fillet, *S. molesta* and commercial feed revealed that fish contained cholesterol (Rf= 0.94), triacylglycerols (TAGs) (Rf= 0.69) and diacylglycerols (DAGs) (Rf=0.51). TAGs constituted a large portion of the lipids in commercial diet.

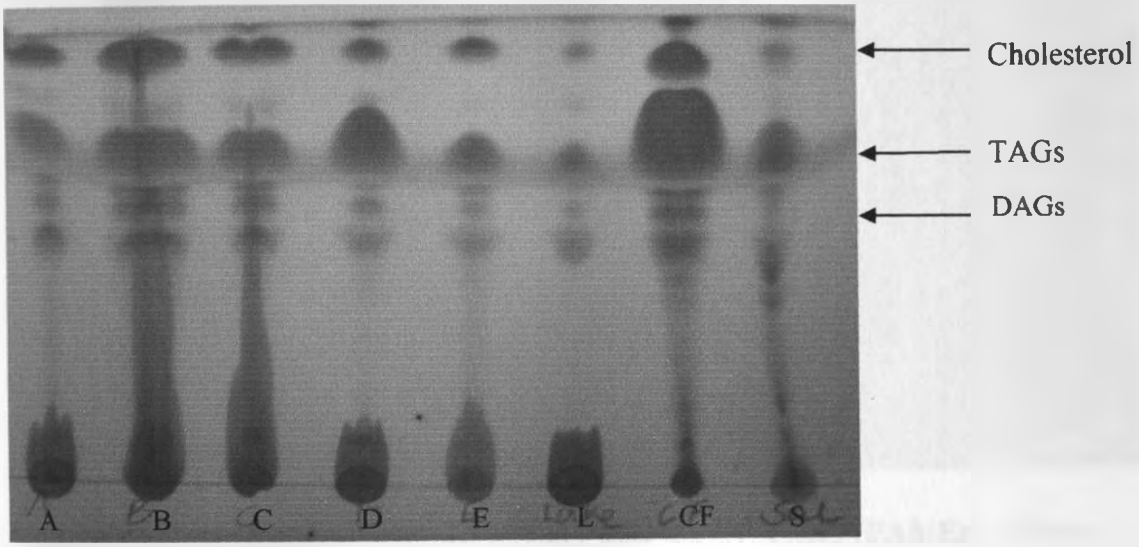


Figure 5: Thin layer chromatography showing the lipid classes in fish and feed samples

A-D represents lipids from fish fed the various diets: A= control (commercial feed), B= 10% *Salvinia molesta*, C= 20% *Salvinia molesta*, D=30 % *Salvinia molesta*,

E= baseline sample of fish (sample taken before the feeding trial commenced)

L= fish sample obtained from the lake (these fish were of similar weight as experimental fish at the end of the feeding trial)

CF = commercial feed

S= *Salvinia molesta*

A thin layer chromatography after transesterification showed a single band per sample. This is indicative of complete esterification.

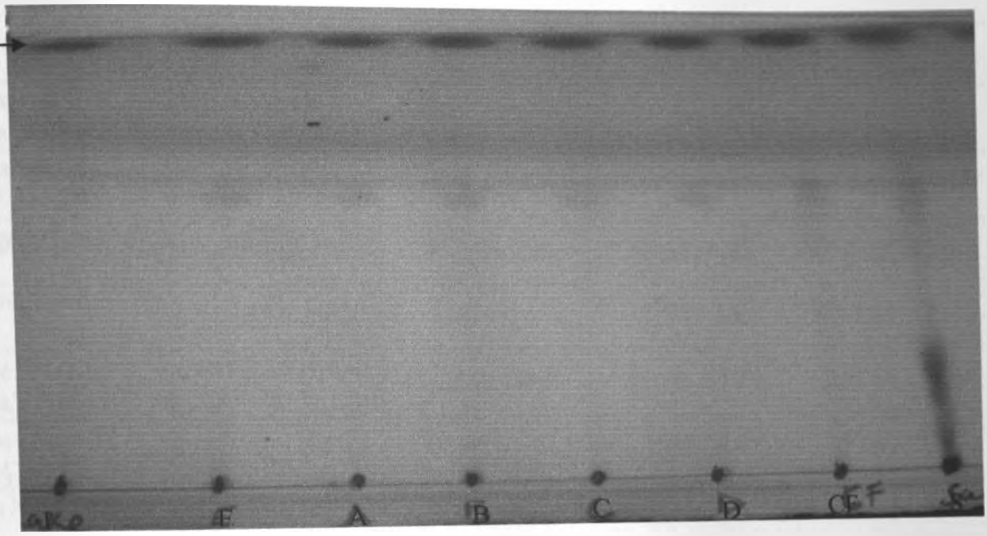


Figure 6: Thin layer chromatogram of fatty acid methyl esters (FAMEs). (Arrow pointing FAME bands)

The letters L, E, A, B, C &D represent the various fish groups; L= sample obtained from the lake; E= baseline sample, A= control (commercial feed), B= 10% *Salvinia molesta*, C= 20% *Salvinia molesta*, D=30 % *Salvinia molesta*, S= *Salvinia molesta.*

CF = commercial feed sample and S = *S. molesta*

Table 6: Growth performance and feed utilization efficiency of fish receiving *Salvinia molesta* at various inclusion levels

	Feed treatment			
Performance parameter	Commercial feed (Control)	10% *Salvinia molesta*	20% *Salvinia molesta*	30% *Salvinia molesta*
mean weight gain(g)[a]	1.46±.12	1.34±.16	1.11±.14	1.27±.13
length gain (mm)	28.63±1.07	25.33±1.4	20.65±4.75	22.37±.20
Feed conversion Ratio [b]	1.63±.13	1.61±.14	1.76±.36	1.81±.23
Relative Growth Rate (%) [c]	447.33±104.48	456.66± 49.83	430.00±177	315.67±55.34
Specific Growth Rate (%)[d]	2.92±.32	3.00±.16	2.82±.61	2.47±.23
Protein Efficiency Ratio [e]	4.42±.38	4.33±.35	4.00± .82	3.71±.49
Mortality (%)[f]	2 ± 1.0^{x}	2.33 ± 1.33^{x}	$12\pm.00^{y}$	$6.5\pm.5^{x}$

Figures here represent the mean ±SE

There was no significant difference between groups for all the growth and feed utilization parameters.

The number of fingerlings that died in the group receiving 20% *S. molesta* differed significantly from the other groups

[a] Weight gain = mean final body weight (g) – mean initial body weight (g)

[b] Feed conversion ratio (FCR) = total dry weight of feed given (g)/wet weight gain (g)

[c] Relative Growth Rate (RGR) =100* [(mean final weight-mean initial weight)/mean initial weight]

[d] Specific growth rate (SGR, %/day) = 100*[(Ln(mean final body weight) – Ln(mean initial body weight)]/culture period (days)

e Protein efficiency ratio (PER) = wet weight gain (g)/crude protein fed (g)

[f] Data on mortality represents average mortality from all the three replicates per treatment

Table 6: Growth performance and feed utilization efficiency of fish receiving *Salvinia molesta* at various inclusion levels

	Feed treatment			
Performance parameter	Commercial feed (Control)	10% *Salvinia molesta*	20% *Salvinia molesta*	30% *Salvinia molesta*
mean weight gain(g)[a]	1.46±.12	1.34±.16	1.11±.14	1.27±.13
length gain (mm)	28.63±1.07	25.33±1.4	20.65±4.75	22.37±.20
Feed conversion Ratio [b]	1.63±.13	1.61±.14	1.76±.36	1.81±.23
Relative Growth Rate (%) [c]	447.33±104.48	456.66± 49.83	430.00±177	315.67±55.34
Specific Growth Rate (%)[d]	2.92±.32	3.00±.16	2.82±.61	2.47±.23
Protein Efficiency Ratio [e]	4.42±.38	4.33±.35	4.00± .82	3.71±.49
Mortality (%)[f]	2 ± 1.0^{x}	2.33 ± 1.33^{x}	$12\pm.00^{y}$	$6.5\pm.5^{x}$

Figures here represent the mean ±SE

There was no significant difference between groups for all the growth and feed utilization parameters.

The number of fingerlings that died in the group receiving 20% *S. molesta* differed significantly from the other groups

[a] Weight gain = mean final body weight (g) – mean initial body weight (g)

[b] Feed conversion ratio (FCR) = total dry weight of feed given (g)/wet weight gain (g)

[c] Relative Growth Rate (RGR) =100* [(mean final weight-mean initial weight)/mean initial weight]

[d] Specific growth rate (SGR, %/day) = 100*[(Ln(mean final body weight) – Ln(mean initial body weight)]/culture period (days)

e Protein efficiency ratio (PER) = wet weight gain (g)/crude protein fed (g)

[f] Data on mortality represents average mortality from all the three replicates per treatment

Fatty Acid Composition of Fish Muscle Lipid

For all the fatty acids except Hexadecanoate and DHA, there was no significant difference between fish receiving the commercial control diet and those receiving *Salvinia molesta* at different inclusion levels. There was also no significant difference between the initial fish (baseline) and any of the treatment groups after 56 days of feeding. The difference in the fatty acid concentrations between the experimental fish and fish of the same size caught from the lake was however significant. Total SFA for the experimental fish was in the range of 414-462µg/mg of total lipid while MUFAs were in the range of 292-304µg/mg of total lipid.

Linoleate was the most abundant n-6 fatty acid in all the feed treatment groups, followed by arachidonate and docosatetraenoate respectively. In contrast ARA was the most abundant FA in lake fish. Total n-6 PUFAs were in the range of 202-235µg/mg of total lipid for the experimental fish, while it was 131 µg/mg for lake fish. DHA was the most abundant n-3 FA (16-31 µg/mg) in all the treatment groups and its level increased with increase in the amount of *S. molesta* in the diet. The n-6: n-3 ratio was highest (6:1) in fish receiving the commercial control diet and lowest in the fish receiving 30% *S. molesta* in their diet. Fish from the lake had an n-6: n-3 ratio of 1:1 and had a much higher total omega-3 concentration compared to the experimental fish (Table 7). There was much more LA than ALA in all the fish groups (Fig. 8).

Table 7: Muscle fatty acid concentration (µg/mg of oil) of fish fed at varied inclusion levels of *Salvinia molesta*

	Baseline	Control	10% *S. molesta*	20% *S. molesta*	30% *S. molesta*	Lake
SFA						
Tetradecanoate	40.7±2.3[a]	37.7±0.8[a]	44.8±1.9[a]	36.8±1.6[a]	37.5±1.1[a]	17.5±2.0[b]
Pentadecanoate	6.5±1.14	4.4±1.7	6.6±.3	5.6±.26	6.4±.3	8.7±.3
Hexadecanoate	327.4±15.1[a]	319.6±1.3[a]	344.1±9.4[ab]	308.4±1.1[a]	322.2±5.4[a]	374.5±2.1[b]
Stearate	77.8±31.2	65.9±20.0	66.9±8.4	87.3±4.2	48.4±8.2	135.8±1.6
Total SFA	**452.4±12.7[a]**	**427.5±17.9[a]**	**462.4±3.3[a]**	**438.1±5.0[a]**	**414.5±4.1[a]**	**536.5**±1.6[b]
MUFAs						
Palmitoloate	59.9±7.5	55.4±9.6	66.6±6.3	43.2±3.9	59.6±10.6	56.4±1.8
Oleate	229.7±18.8[a]	237.9±32.7[a]	222.4±32.2[a]	250.7±7.3[a]	222.4±26.9[a]	83.8±.9[b]
Vaccenate	ND	ND	ND	ND	ND	58.3±2.0
Eicosenoate	6.1±.7	10.12±1.4	8.2±1.9	10.9±1.4	8.54±.2	5.0±.0
Erucate	1.2±1.2	ND	1.2±1.2	ND	1.8±1.8	3.2±.1
Total MUFAs	**296.8±26.8**	**303.5±40.9**	**298.3±37.8**	**304.7±12.6**	**292.3±39.1**	**206.7**±4.5
n-6 PUFAs						
Linoleate	170.9±18.9[a]	212.6±21.4[a]	183.9±25.3[a]	197.8±14.8[a]	207 8±27.3[a]	47.5±1.9[b]
Arachidonate	18.5±3.8[a]	13.5±.9[a]	14.2±2.4[a]	15.0±1.9[a]	21.1±4.1[a]	62.1±1.1[b]
Docosatetraenoate	3.4±.9	4.8±.2	4.1±1.3	4.5±.7	6.7±1.7	22.2±3.0
Total n-6 PUFAs	**192.9±23.6**	**230.9±22.5**	**202.2±29.0**	**217.3±17.5**	**235.5±33.0**	**131.8**±.0
n-3 PUFAs						
Linolenate	10.9±2.0	9.7±1.0	7.5±.1	10.8±1.8	11.3±.3	23.4±7.8
Octadecatetraenoate	12.7±10.4	1.4±1.4	1.0±1.0	ND	1.4±1.4	5.2±.2
Eicosapentaenoate	2.8±.4[a]	4.2±1.6[a]	3.9±.8[a]	3.6±.2[a]	4.8±2.1[a]	20.2±4.9[b]
Docosapentaenoate	2.9±.5[a]	6.9±1.7[a]	4.9±.6[a]	5.4±.9[a]	8.9±1.9[a]	17.1±3.0[b]
Docosahexaenoate	28.7±7.3[a]	15.9±2.7[a]	19.9±5.3[a]	20.1±2.6[a]	31.4±7.2[ab]	59.1±1.9[b]
Total n-3 PUFAs	**58.0±15.8**	**38.1±.5**	**37.2±5.6**	**39.9±.12**	**57.7±10.1**	**125.0±7.5**
n-6:n-3	**4:1**	**6:1**	**5:1**	**5:1**	**4:1**	**1:1**

Values here represent mean ±S.E. ND= not detectable (value<0.05µg/mg)
Values in the same row that have different superscript letters differ significantly from each other ($p \leq 0.05$). Baseline= fish sample taken prior to starting the feeding trial; Lake= Fish caught from lake Baringo at the end of the feeding trial (they were equal of similar size as the experimental fish at the end of the feeding trial)

For all the fish groups, SFAs were the highest making up nearly half of the fatty acids. This was followed by MUFAs then n-6 PUFAs and least was n-3 PUFAs (Figure 7).

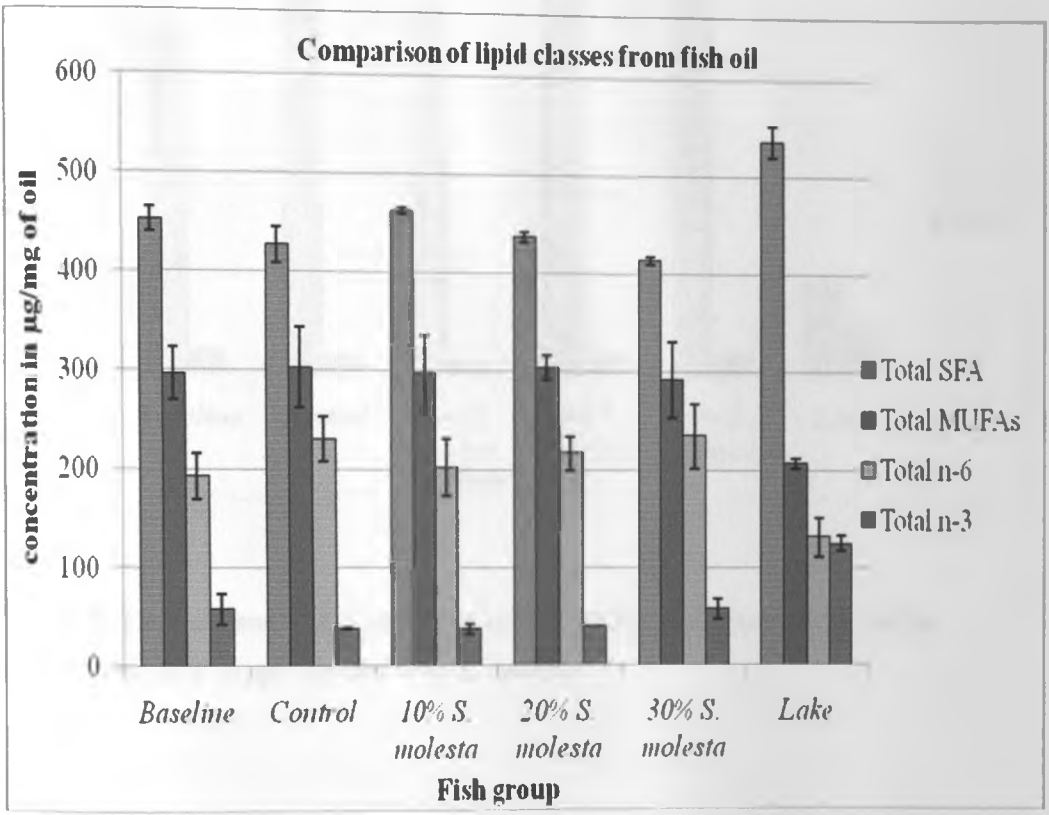


Figure 7: Concentrations of the various fatty acid classes in the fish maintained on the various experimental diets.

Baseline= fish sample taken before commencement of feeding trial

Lake= sample taken from Lake Baringo, these were of a similar size as the experimental fish at the end of the feeding trial.

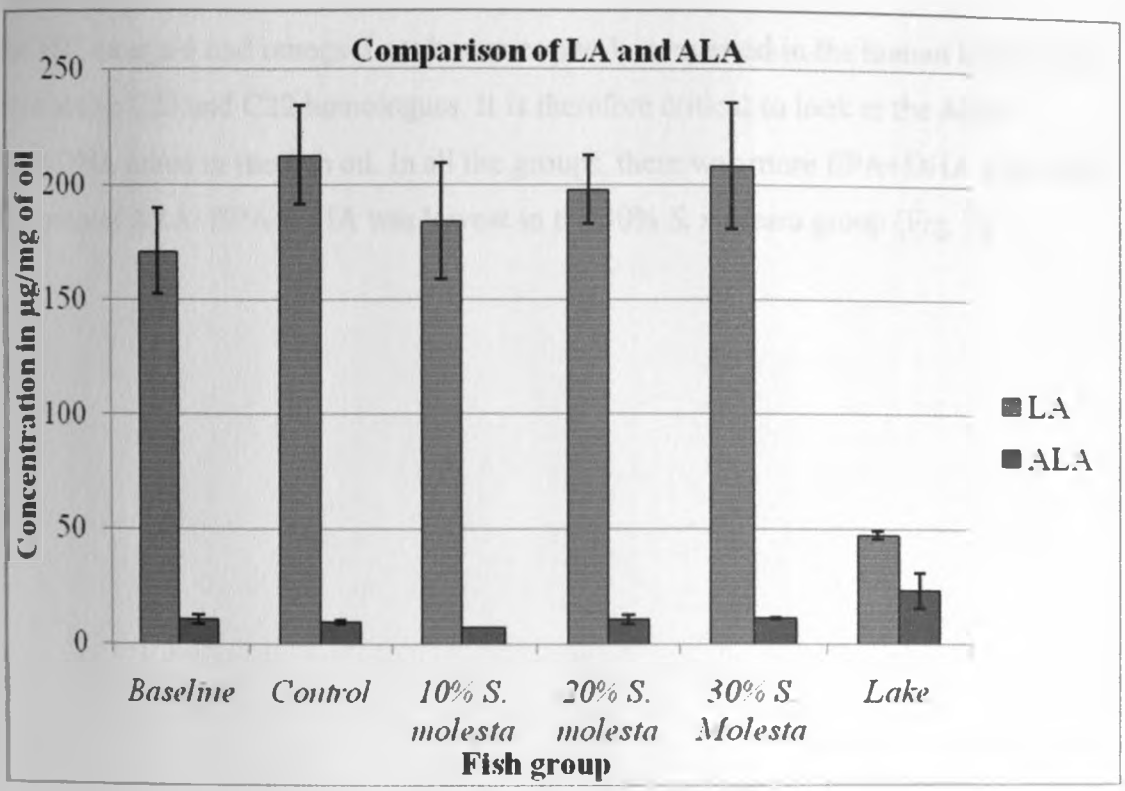


Figure 8: Comparison of LA and ALA in fish oil derived from tilapia fed on commercial feed supplemented with *S. molesta*

The 18C omega-6 and omega-3 are however poorly converted in the human body to the more active C20 and C22 homologues. It is therefore critical to look at the ARA: EPA+DHA ratios in the fish oil. In all the groups, there was more EPA+DHA than ARA. The ratio of ARA: EPA+DHA was lowest in the 30% *S. molesta* group (Fig. 9)

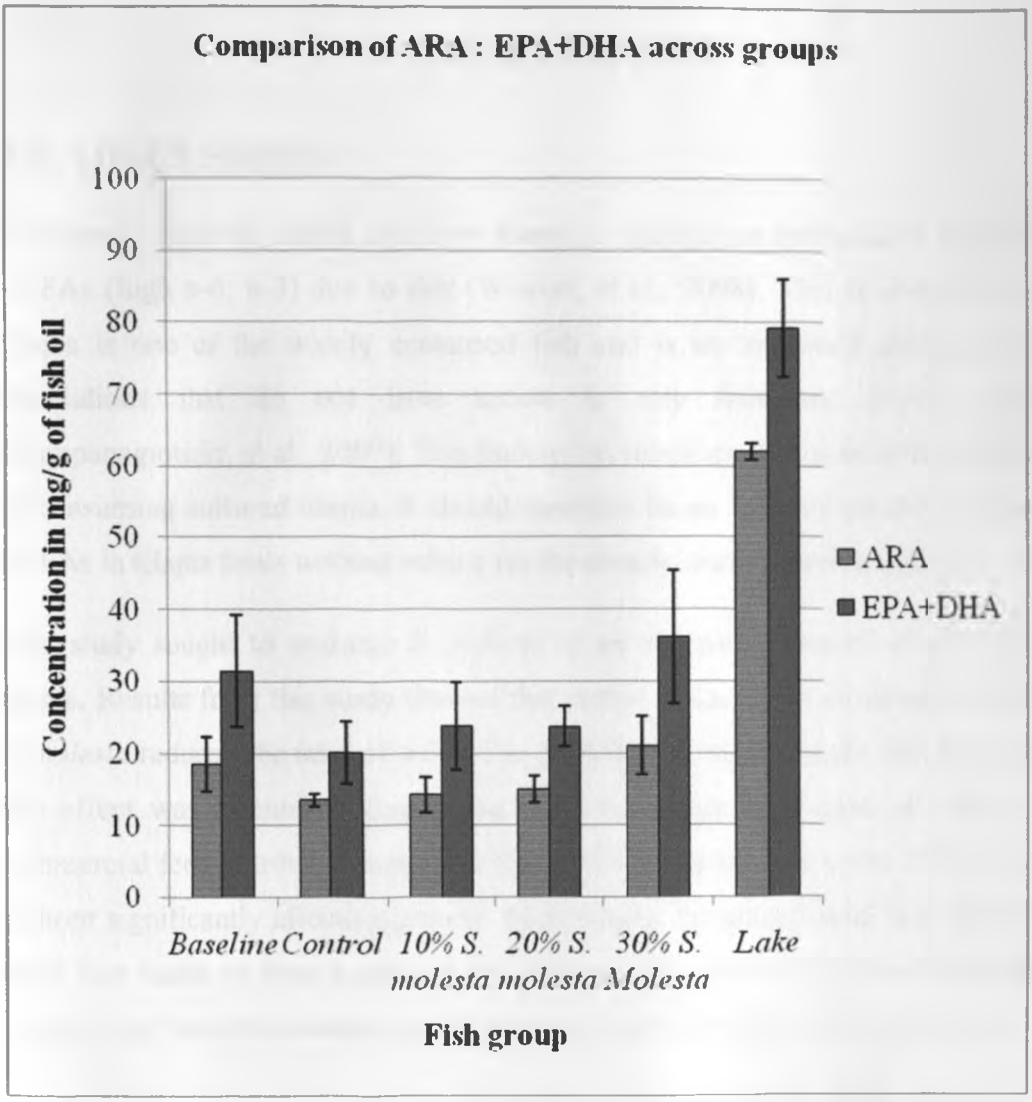


Figure 9: Comparison of ARA and EPA+DHA levels in fish oil across groups.

In all the experimental groups as well as the baseline and fish sample from the lake, the total EPA+DHA was found to be higher the ARA content.

CHAPTER FIVE

)ISCUSSION

vely cultured tilapia has been found to contain an unfavorable combination of s (high n-6: n-3) due to diet (Weaver, et al., 2008). This is despite the fact that is one of the widely consumed fish and is an important dietary resource for ıtions that do not have access to oily fish and PUFA supplements)anagiotidis, et al., 2007). This finding has raised questions as to the health benefits ısuming cultured tilapia. It should therefore be an industry priority to increase n-3 s in tilapia feeds without relying on the already over-exploited fish oil.

study sought to evaluate *S. molesta* as an alternative source of n-3 for cultured ı. Results from this study showed that partial replacement of commercial feed with *lesta* reduced the ratio of n-6: n-3 in both feed as well as in the fish fillet. However, :ffect was minimized due to the overwhelmingly high ratio of n-6: n-3 in the nercial feed. Further, commercial feed could be replaced by up to 30% by *S. molesta*)ut significantly affecting growth. Interestingly, the commercial feed chosen for this was found to have a much lower protein concentration than was specified by the lier and hence the overall growth was lower than would have been expected.

Proximate composition of commercial feed, *S. molesta* and erimental diets

ılts from this study revealed that *S. molesta* has a high content of ash (31.63%) and e fiber (18.30%) while the level of crude protein was 18.3% (Table 2). Comparable lts were obtained by other researchers analyzing *S. molesta* from India who obtained .3% crude protein and 18.5% crude fiber (Hasan & Chakrabarti, 2009; Moozhiyil & auf, 1986). On the other hand, the commercial feed used in the study was found to e unhealthy levels of starch and crude sugar content (NFE, 52.2%). Furthermore, the tein content was only 14.95% which is significantly lower than the 37% percent icated on the supplier's label. Similar observations were reported by Nalwanga and

colleagues who found that some commercial fish feeds in East Africa had lower levels of nutrients than indicated on the label (Nalwanga, Liti, Waidbacher, Munguti, & Zollitsch, 2009). In general the commercial control diet failed to meet the recommended nutrient requirement for tilapia fingerlings. Tilapia fingerlings require a protein content of at least 40% and at least 8% lipid on dry matter basis (Mjoun, et al., 2010).

The experimental diets were constituted from the commercial feed and *S. molesta* at various ratios. The amount of crude protein and crude fiber in the *S. molesta* supplemented test diet increased though not significantly (Table 3). Likewise, total lipid, NFE and moisture content were not significantly affected by inclusion of *S. molesta* in the test diet. The amount of ash was however significantly lower in the commercial feed compared to diets supplemented with *S. molesta* ($p<0.05$).

5.2. Growth response

Growth rate is mainly dependent on the protein content of the feed and diets with higher protein generally yield higher growth rates. As expected the growth rate across the treatment groups was not significantly different just as the protein content in the diets (Table 6). Furthermore, growth was not affected by inclusion of *S. molesta*. The group receiving 20% *S. molesta* recorded the highest mortality (12%). This could be attributed to other factors other than diet.

5.3. Fatty acid composition of commercial feed, *S. molesta* and experimental diets

The commercial feed had a high LA content (50.61%). In contrast there was no detectable amount of ARA, this would be expected if the feed ingredients were mainly of terrestrial plant origin. Commercial feed also contained ALA, EPA, DPA and DHA in minute amounts. The ratio of omega-6 to omega-3 was much higher in commercial feed (32:1) compared to 0.56:1 in *S. molesta*. Hexadecanoate was the major fatty acid in *S. molesta* constituting 34.93% of the total fatty acids while the amount of LA and ALA were almost equal. *S. molesta* also contained ARA, EPA, DPA and DHA. Inclusion of *S. molesta* in the experimental diets introduced a considerable amount of omega-3. On the

r hand the amount of ARA in the experimental diets stemming from *S. molesta* was stically significant as compared to the commercial feed

. Lipid Content and Fatty acid composition of fish fillet

ıpia is generally a low lipid fish (<1% in wet fillet) compared to others like salmon ırapanagiotidis, et al., 2007). Results obtained in this study after 56 days of feeding ıfirm these observations. The fillet lipid content of the experimental fish was in the ıge of 0.96-1.43% of wet fillet. The fish receiving the commercial control diet had the ghest lipid content while those receiving 30% *S. molesta* had the least, thus reflecting ɜ dietary intake. The fatty acid composition of fish fillet was further reflective of the ɛtary intake. The elevated SFA in the diet yielded high SFA in the fish fillet. This is in greement with findings by other scientists that the lipid and fatty acid profile of fish is ɛpendent on diet (Grisdale-Helland & Helland, 1997; Hardy & Lee, 2010; Jobling & ɔhansen, 2003).

ɹt the end of the experimental period, fish from all the experimental groups excluding vild lake fish were found to have a high LA: ALA ratio. This could be due to the fact that ırior to the feeding trial the ratio was already high and likewise was the ratio of the ɛxperimental diets. On the other hand, at 30% *S. molesta* inclusion there was a ɜignificantly higher level of DHA in the fillet ($p<0.05$). Further, inclusion of *S. molesta* in the experimental diets introduced ARA in both the feed and fish fillet. The level of ARA was however still lower in fish receiving *S. molesta* compared to the wild lake fish. In general inclusion of *S. molesta* in the fish diets had a positive effect on the n-6: n-3 ratio. It thus could be said that fish whose diet were supplemented with *S. molesta* is a healthier source of fatty acids compared to that fed commercial feed only.

5.5. Conclusion

Salvinia molesta has showed great potential to be utilized as an omega-3 source for cultured tilapia. Its partial inclusion in the experimental diets lowered the omega-6 to omega-3 ratio not just in the diet but also in the fish fillet. Results in this study were however overshadowed by the high content of linoleic acid (n-6) in the commercial control diet.

This study also found that the protein content in the commercial control diet was much lower than indicated on the label. Mixing such feed with locally available plant material to cut feed costs results in an even poorer diet which in turn greatly affects growth of fish and lower returns for fish farmers. The government of Kenya through the Kenya Bureau of Standards (KEBS) should ensure quality feeds for farmers.

5.6. Recommendations

This study has provided evidence that *S. molesta* is a good and potential source of omega-3 for cultured tilapia. In areas where it grows as a weed, fish farmers can collect dry it and use it as a feed supplement or feed manufacturers can utilize it as an ingredient. However, before the plant can be fully utilized it will be important to conduct research as to the anti-nutritive elements that the plant may contain.

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7.0. APPENDICES

Appendix 1: List of fatty acid standards

Fatty acid	Acronym
Internal std	C 13:0
Methyl tetradecanoate	C14:0
Methyl pentadecanoate	C 15:0
Methyl hexadecanoate	C 16:0
Methyl Palmitoloate	C 16:1
Methyl Stearate	C 18:0
Methyl oleate	C 18:1
Methyl Vaccenate	C 18:1
Methyl linoleate	C 18:2
Methyl linolenate	C 18:3
Methyl octadecatetraenoate	C 18:4
Methyl eicosenoate	C 20:1
Methyl Arachidonate	C 20:4
Methyl Eicosapentaenoate	C 20:5
Methyl Erucate	C 22:1
Methyl docosatetraenoate	C 22:4
Methyl docosapentaenoate	C 22:5
methyl docosahexaenoate	C 22:6

Appendix 2: Sample Chromatograms

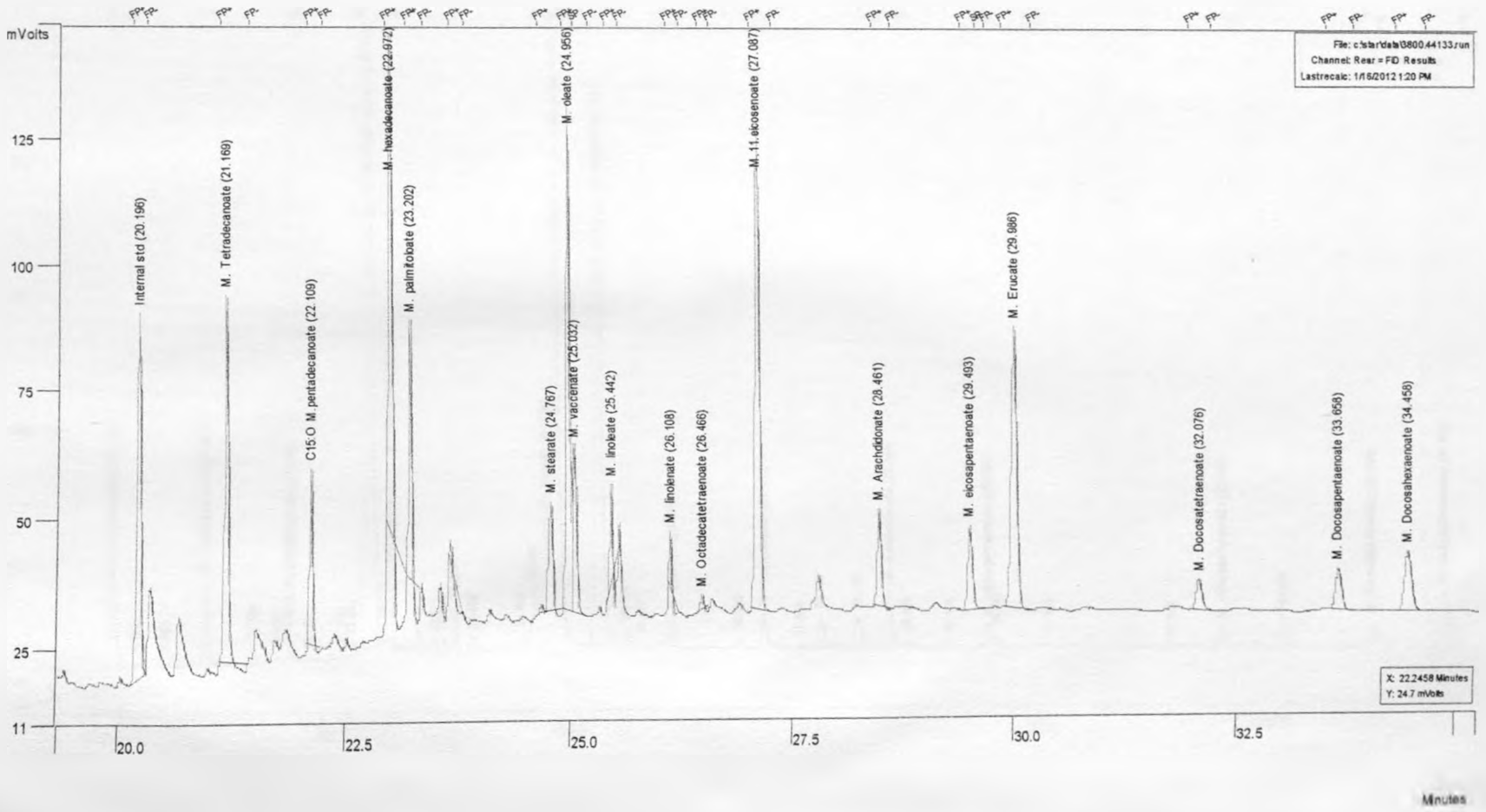

Figure 10: Standard mixture chromatogram

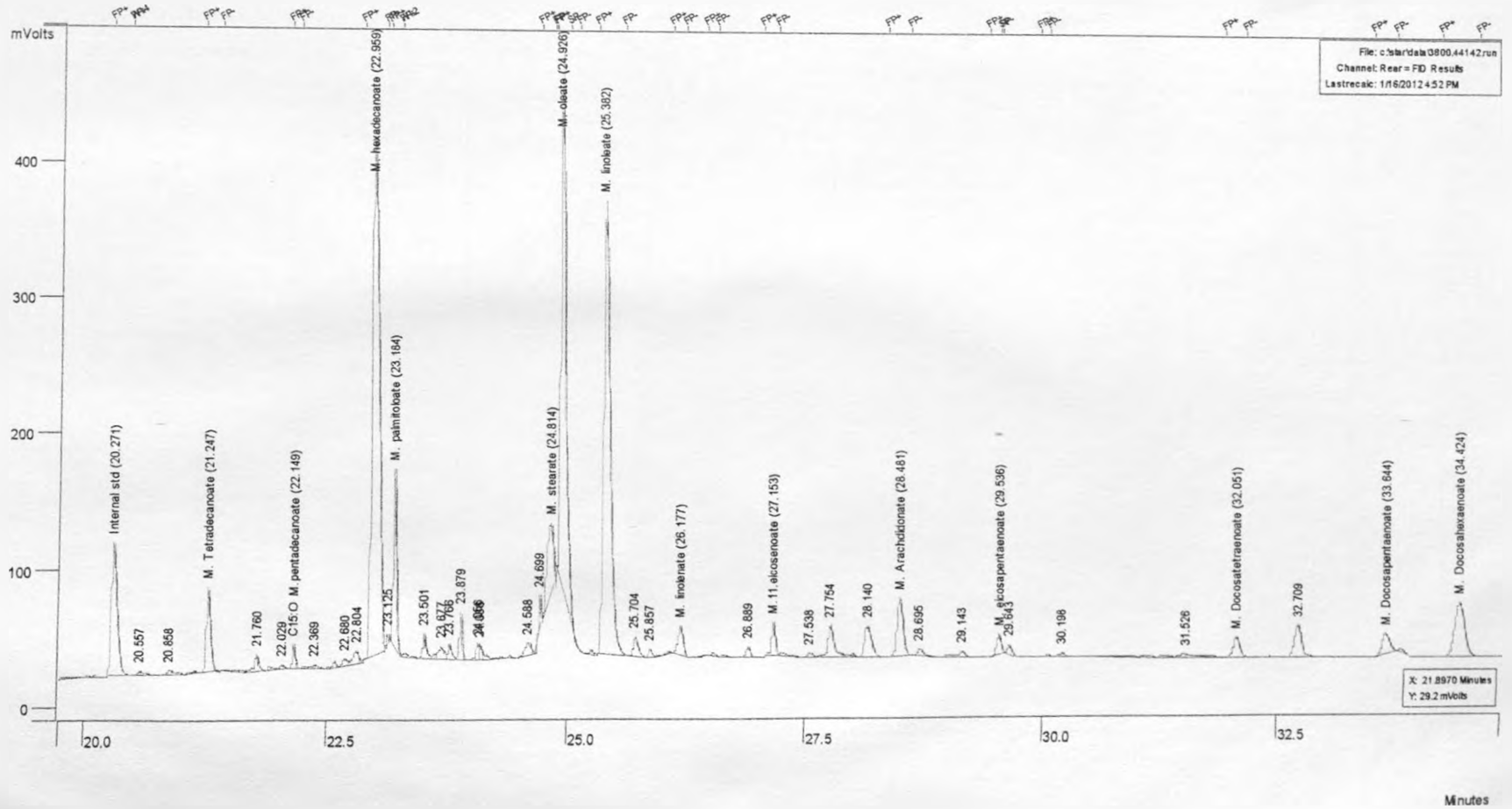

Figure 11 : Fatty acid profile of fish oil from fish receiving 30% *S. molesta* in their diet