EFFECT OF PHYTOGENIC FEED ADDITIVES ON GONADAL DEVELOPMENT IN MOZAMBIQUE TILAPIA (*OREOCHROMIS MOSSAMBICUS*)

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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own original work, that I am the owner of the copy right thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part, submitted it for obtaining any qualification.

Signature:

Date: February 12, 2013

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Summary

This study investigated the "Effect of Phytogenic Feed Additives on Gonadal Development in Mozambique tilapia (*Oreochromis mossambicus*). Aquaculture remains the fastest growing animal food-producing sector and it is set to overtake capture fisheries as a source of food fish, and also to outpace population growth. This rapid growth can be attributed to the increasing demand for aquaculture products, and an urgent need for a sustainable food resource and the safe production of food. Globally, fish provides over 3.5 billion people with up to 15 percent of their average per capita intake of animal protein.

The introduction of aquaculture to sub-Saharan African (SSA) took place during the 1940s and 1950s, and the main objectives were to improve nutrition in rural areas, allow for the diversification of activities to reduce the risk of crop failures, to generate additional income, and to create employment opportunities. Aquaculture was seen as a viable option for rural development in SSA, and substantial resources were invested to support its development. Poor results were however, recorded in terms of production and sustainability.

Tilapia is the most widely cultured of all fish species farmed, and the second most important group of freshwater and brackish water fish after carps. Tilapia has all the necessary traits that makes it an excellent species to culture, but cost-efficient production is hampered by the animals attaining sexual maturity at a an early age, which then result in precocious breeding in aquaculture systems. The production of single-sex populations (i.e. all male) is a potential means to address the problem of precocious breeding, but the technologies used to establish single-sex populations are not readily available to resource-poor communities that farm with tilapia for food purposes.

Phytochemicals, also known as phytoestrogens, are plant-derived compounds that structurally or functionally mimic mammalian estrogens that affect the sexual differentiation of fish. Phytochemicals occur in plants like Pawpaw (*Carica papaya*) and Moringa (*Moringa oleifera*). The study thus had a threefold objective. Firstly, the study investigated the potential of Pawpaw seed meal (P) and Moringa seed meal (M), as part of a commercial tilapia diet, to be used as endocrine disrupting compounds (EDC's) to control the reproduction of sexually mature Mozambique tilapia (20-45g). Secondly, assess the potential of P and M to inhibit the attainment of sexual maturity in immature tilapia (2-8g). Finally, P and M to determine its influence on sexual differentiation of tilapia fry (9-12 days posthatch) to produce all-male populations.

The study indicated that both P and M seeds contain bio-active chemicals that are capable of disrupting the gonad function, differentiation and sexual maturation of Mozambique tilapia. Sperm production was affected, evident in the degeneration of the testicular tissue samples. Egg production, ovulation and spawning were all affected, as evident in the difference in colour of the degrading eggs, as well as the absence of spawning. Ovo-testes were observed in cases where diets containing 10.0g P and 10.0g M /kg basal diet were fed. Eggs were observed in the ovaries of sexually immature fish, but spawning did not occur. The study also presents the first report on the isolation of Oleanolic acid in Moringa seeds.

The evident of antifertility properties of both Pawpaw and Moringa seeds can be exploited to control or prevent reproduction of Mozambique tilapia in SSA aquaculture systems. This could be of particular importance to aquaculture development in rural areas of Sub-Saharan African countries, given the abundant year round availability of these compounds. Further studies are required to optimise the preparation of the experimental compounds; as well as determining the optimal inclusion level of the phytogenic compounds, as well as how their efficacy to manipulate the reproductive potential and ability of Mozambique tilapia are influenced by environmental factors such as water temperature.

Opsomming

Akwakultuur is die vinnigste groeiende dierlike voedsel-sektor. Daar word verwag dat dit visserye sal oortref as 'n bron van voedsel en dat groei in dié bedryf selfs die bevolkingsgroei sal verbysteek. Die vinnige groei in die sektor kan toegeskryf word aan die toenemende vraag na akwakultuur produkte en 'n dringende behoefte vir 'n volhoubare voedsel hulpbron, wat ook die veilige produksie van voedsel sal verseker. Wêreldwyd voed vis meer as 3.5 miljard mense en dra tot 15% van die gemiddelde hoeveelheid dierlike proteïen per kapita ingeneem, by.

Die bekendstelling van akwakultuur in sub-Sahara Afrika (SSA) het gedurende die 1940's en 1950's plaasgevind, met die belangrikste doelwitte om voeding in landelike gebiede te verbeter, geleenthede vir diversifisering te skep wat die risiko van misoeste verminder, om bykomende inkomste te genereer en werksgeleenthede te skep. Akwakultuur is gesien as 'n lewensvatbare opsie vir die ontwikkeling van die landelike gebiede in SSA en aansienlike hulpbronne is belê om die ontwikkeling daarvan te ondersteun. Swak resultate is egter in terme van produksie en volhoubaarheid behaal.

Tilapia is die mees algemene spesies wat gekweek word en is die tweede mees belangrike groep van varswater en brak water vis soesies, na Karp. Tilapia beskik oor al die nodige eienskappe wat dit 'n uitstekende spesie vir voedselproduksie maak, maar koste-doeltreffende produksie daarvan word gekortwiek deur die feit dat die spesie seksuele volwassenheid op 'n vroeë ouderdom bereik, wat dan lei tot vroeg-rype teling en die gevolglike oorbevolking en swak groei van tilapia in 'n akwakultuur sisteem. Die produksie van enkel-geslag bevolkings (d.i. slegs manlike vis) is 'n potensiële oplossing vir dié probleme, maar die tegnologie wat gebruik word om enkel-geslag bevolkings te produseer is nie geredelik toeganklik vir hulpbron-arme gemeenskappe wat met Tilapia vir kosdoeleindes boer nie.

Fitochemikalieë, anders ook bekend as fito-estrogene, is verbindings wat in plante voorkom en wat struktureel of funksioneel die werking van die natuurlike soogdier estrogene/androgene naboots, met die fitochemikalieë wat die seksuele differensiasie van vis beïnvloed. Fitochemikalieë kom in plante soos papaja (*Carica papaya*) en Moringa (*Moringa oleifera*) voor. Die studie het dus gepoog om die potensiaal van papaja saad meel (P) en Moringa saad meel (M), as deel van 'n kommersiële tilapia dieet, om as endokriene ontwrigters (EDC's) gebruik te word om die reproduksie van seksueel volwasse Mosambiek tilapia (20-45g) te beheer, om te voorkom dat onvolwasse tilapia (2-8g) geslagsrypheid te vroeg bereik en ook om die geslagsdifferensiasie van tilapia vingerlinge (9-12 dae na uitkom) te manipuleer om enkel-geslag (manlike) produksiegroepe te produseer.

Die studie het aangedui dat beide papaja en Moringa sade bio-aktiewe chemikalieë wat die werking van die gonadotrofien hormone, geslagsdifferensiasie die stadium waarop Mosambiek tilapia geslagsrypheid bereik, kan beïnvloed. Spermproduksie is negatief beïnvloed, soos waargeneem in die degenerasie van die testisweefsel. Eierproduksie, ovulasie en die vrystelling van eiers is almal negatief beïnvloed, soos duidelik waargeneem in die kleurverskil (van normale eiers) van eiers wat 'n mate van reabsorbsie aandui en die feit dat geen eiers vrygestel is nie. Ovo-testes is waargeneem in gevalle waar diëte met 10.0g papaja en / of Moringa / kg basale dieet gevoer is. Eiers is waargeneem in die eierstokke van seksueel onvolwasse vis, maar vrystelling het nie plaasgevind nie. Die studie is die eerste verslag oor die isolasie van Oleanoliese suur in Moringa sade.

Die duidelike reproduksie-beperkende (anti-vrugbaarheid) eienskappe van beide papaja en Moringa sade kan benut kan word om reproduksie in Mosambiek tilapia te voorkom of te manipuleer in SSA akwakultuur stelsels. Dit is veral van besondere belang vir akwakultuur ontwikkeling in die landelike gebiede van SSA lande, gegewe dat beide sade regdeur die jaar geredelik beskikbaar is.

Verdere studies word benodig om protokolle vir die voorbereiding van die eksperimentele verbindings te optimaliseer, sowel as die bepaling van die optimale insluitingsvlakke van die fitogeniese verbindings, asook hoe die doeltreffendheid van hierdie verbindings deur omgewingsfaktore soos water temperatuur beïnvloed word.

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Chapter 1

General Introduction

1.1 Introduction

Aquaculture can be defined as, “the farming of aquatic organisms, which include fish, crustaceans, molluscs, and aquatic plants in freshwater, brackish-water and sea water environments” (FAO, 1999, El-Sayed, 2006). Aquaculture is believed to have its origin in China in the fifth century (Pillay and Kutty, 2005). It is reported that oysters have been farmed inter-tidally in Japan some 3000 years ago, and also by the Romans nearly 2000 years ago (Stickney, 2005). The growth in aquaculture, however, blossomed within the not too distant decades, threby becoming one of the world’s fastest growing food sector since the mid 1980s on a growth rate of 11 percent annually in comparison to terrestrial farmed meat production and the 1.4 percent of stagnating capture fisheries (FAO, 2002; 2003). Asmah (2008) reported that this rapid growth of the sector can be attributed to the increasing demand for aquaculture products, the urgent need for a sustainable food supply, the increasing scientific, technological and entrepreneurial skill in managing species lifecycles and production environments, thereby generating profit and income, and meeting market and commercial objectives.

1.2 Contribution of Aquaculture to Global Food Consumption

According to FAO (2010) fish supplies approximately 15 percent of average per capita consumption of animal protein to over 3.5 billion people globally. The 2007 figure indicates that gobally, 15.7 percent of average animal protein consumed wwas fish and in terms of all kinds of proteins is pegged around 6.1 percent. In developing countries average per capita supply per annum was 15.1 kg, and 14.4 kg in low-income food-deficit countries (LIFDCs), for example Benin, Chad, Ghana, Zimbabwe, Haiti, Bangladesh, Iraq, Sri Lanka, Kiribati and Republic of Moldova. It is estimated that in LIFDCs, animal protein consumed is very low, however, fish consumption is high sometimes contributing approximately 20.1 percent to total animal protein intake. In the LIFDC’s there is under reporting of statistical informatition, particularly, those from small scale and subsistence fiheries, thereby there is assumption that fish contributes more than it is known on record (FAO, 2010).

In 2008 the total fish supplied world wide from aquaculture and fisheris was approximately 142 million, where about 115million tonnes was used as fish food for human indicating an all-time high per capita supply of about 17 kg (Table 1.1). For 2009 aquaculture production was, estimated at 55.1 million tonnes and 57.2 million tonnes for 2010 (FAO, 2010; 2011).

Currently, in the global animal food production sector aquaculture continue to grow faster than fisheries, livestock, and will eventually overtake the capture fisheries for food fish source, and also outpace population growth (FAO, 2012a). Production of food fish from aquaculture increased at an average annual growth rate

of 8.8 percent in the period of 1980 – 2010 (Figure 1.1), while the world population grew at an average of 1.6 percent per year. The FAO's global forecast considers aquaculture as a fish-food production sector with a huge potential as an income-generating activity, playing an essential role in food security and poverty mitigation.

Table 1.1 Estimated world capture fisheries, aquaculture production and consumption between 2008 and 2010 (adapted from FAO, 2011 and State of World Aquaculture, 2010).

	2008	**2009**	**2010**
Total Production (million tonnes)	142.3	145.1	147.0
Capture fisheries	89.7	90.0	89.8
Aquaculture	52.5	55.1	57.2
Total utilization	142.3	145.1	147.0
Food	115.1	117.8	119.5
Feed	20.2	20.1	20.1
Other uses	7.0	7.2	7.4
Aquaculture contribution			
To total fish output (%)	36.9%	37.9%	38.9%
To food fish output (%)	45.6%	46.8%	47.9%
Per capita food fish consumption (kg/year)	17.1	17.2	17.3
Capture fisheries(kg/year)	9.3	9.2	9.0
Aquaculture (kg/year)	7.8	8.1	8.3

In 2008 aquaculture contributed 52.5 million tonnes (45.6%) to global production of food fish, with a value of US$ 98.4 billion. Estimates from the FAO indicate that by the end of 2012 more than 50% of global food fish consumption will originate from aquaculture. Aquaculture production is predicted to further expand and play an increasingly important role in meeting global fish demands, the acquisition of food security and as an income generating activity. According to Gjedrem *et al.* (2012) if the aquaculture sector continues to expand at its current rate, production will reach 132 million tonnes of fish and shellfish, and 43 million tonnes of seaweed in 2020 (Figure1. 2).

The growth of Aquaculture, however, is not uniformly spread around the world with marked variations between regions and countries in terms of production level, species composition, farming systems and producer profile. According to a FAO report (FAO, 2010) the Asia–Pacific region contributes the bulk of global fisheries production (89.1%), of which China contributes 47.5 million tonnes in 2008 (62.3%), with 32.7 and 14.8 million tonnes respectively from aquaculture and capture fisheries (FAO, 2011).

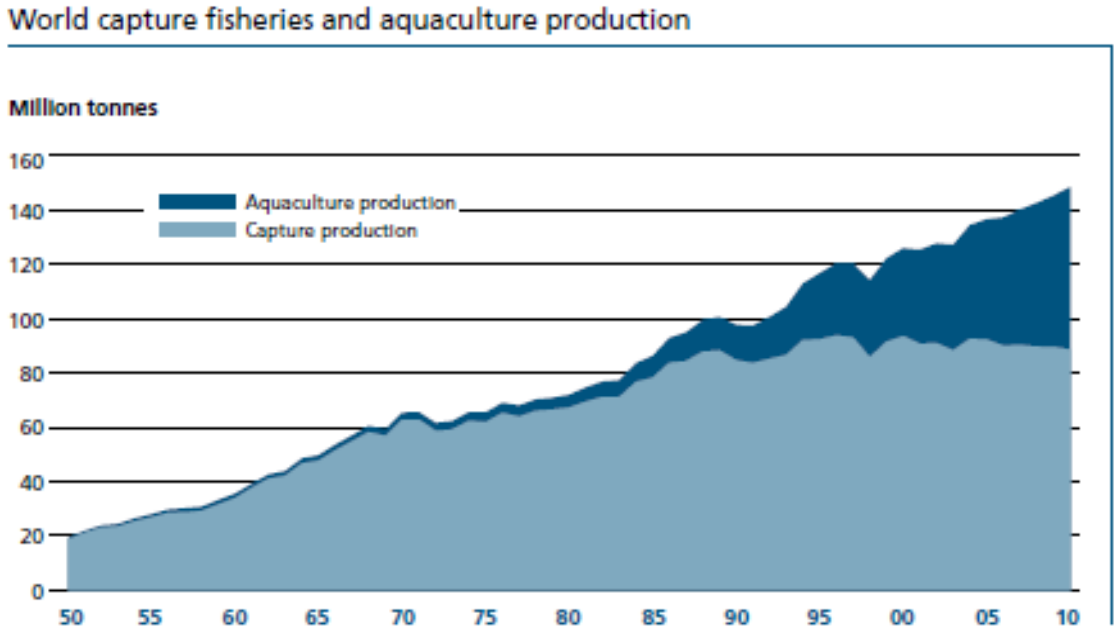

Figure 1.1 World capture fisheries and aquaculture production from 1950-2010 (adapted from State of World Fisheries & Aquaculture, FAO, 2012a).

Moreover, of the 15 leading aquaculture-producing countries, 11 are in the Asia–Pacific region (i.e. China, Thailand, Philippines, Viet Nam, Taiwan - Provence of China, Indonesia, India, Myanmar, Japan, Bangladesh and South Korea). In Africa, only Egypt is listed in the leading 15, with the remainder of the above mentioned countries made up by Norway, Chile and the United States of America.

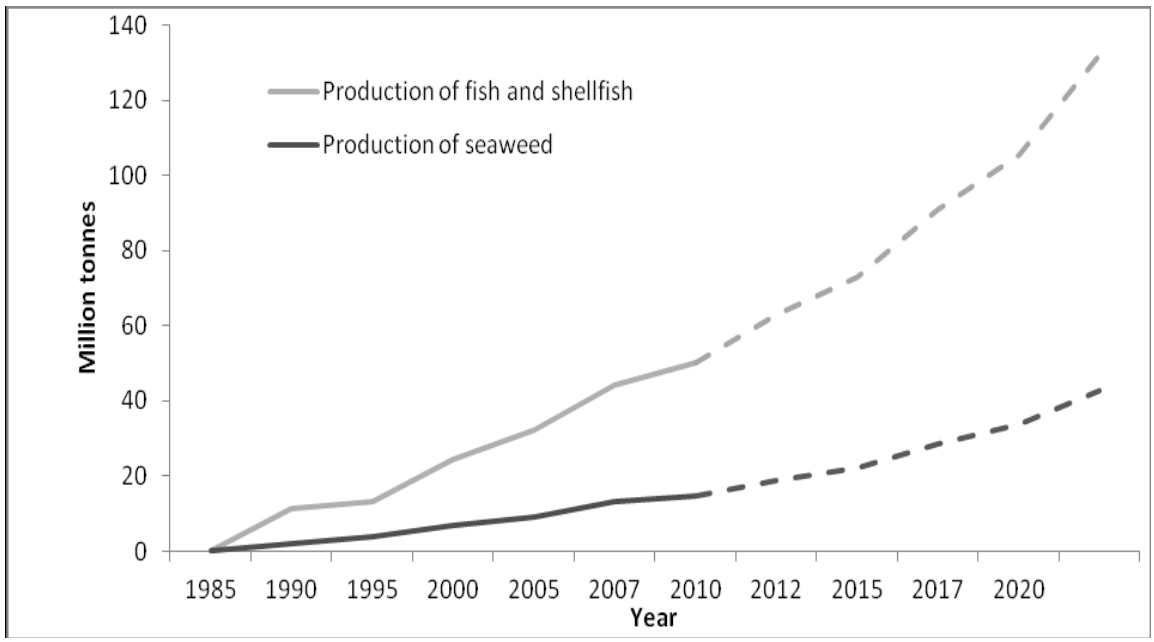

Figure 1.2 Aquaculture production from 1985 to 2007 with projected values (dashed). (Adapted from Gjedrem *et al.,* 2012).

1.3 The role of aquaculture in food security and poverty alleviation

According to FAO (1996) the term food security refers to "a condition when all people, at all times, have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life". This definition includes the nutritional aspect, which is described as access to nutritious food to meet their dietary needs. The term food and nutrition security is also used to emphasise access and appropriate utilisation of micronutrient-rich foods, including the process through which they are cooked and absorbed in the body, and then used in physiologic functions at individual level. Poverty is is directly associated with food insecurity and must be addressed in order to improve access to food. The World Bank (2004) defined poverty as "a multidimensional phenomenon, encompassing inability to satisfy basic needs, lack of control over resources, lack of education and skills, poor health, malnutrition, lack of shelter, poor access to water and sanitation, vulnerability to shocks, violence and crime, lack of political freedom and voice". It is estimated that there are 925 million hungry people in the world and around 1.4 billion people live on less than US$ 1.25 a day, thus living under an extreme economic poverty (IFAD, 2012.)

The contribution of fish to food security and nutrition, is demonstrated in Africa where estimations are for fish to contribute up to 22 percent of the protein intake in Sub-Saharan Africa (FAO, 2011). The relative contribution may be higher than 50 percent in poorer countries due to the scarcity and cost of animal protein sources. In West African coastal countries fish has been a central element in local economies for many centuries and the contribution of fish to dietary protein can be as high as 47 percent in Senegal, 62 percent in Gambia and 63 percent in Sierra Leone and Ghana (World Fish Centre, 2005; FAO, 2012b).

1.4 Aquaculture development in Africa

Apart from production of fish in Egypt that dates back to around 4000 BC (Popma and Lovshin, 1995; El-Sayed, 2006), earlier attempts in Africa include that of tilapia culture in Kenya in 1924 (Maar *et al.*, 1966), the Congo in 1937, Zambia in 1942 and Zimbabwe in 1952 (Satia, 1989).The introduction of aquaculture to Sub-Saharan African (SSA) was done by the colonial powers of Britain, Belgium, France and Portugal in the 1940s and 1950s. The main objectives behind this move were to improve nutrition in rural areas, diversification of activities to reduce risk of crop failures, to generate additional income and the creation of employment. Aquaculture was seen as a viable option for rural development and substantial resources were invested to support its development. Fish culture subsequently spread to other SSA countries, and by the late 1950s up to 300,000 ponds had been constructed; together with several field stations for both research and demonstration/extension purposes (Satia, 1989, Brummett and Williams, 2000; Lazard, 2002; Hecht, 2006).

Poor results were however recorded in terms of production and sustainability followed by a marked decline in fish farming activities in the 1960s (Machena and Moehl, 2000). Lazard (2002) attributed the decline to a general lack of expertise in fisheries science and fish culture. A resurgence of fish culture occurred in the 1970s and 80s as funding agencies invested on a large scale in rural development in many African countries, again with variable success (Stomal and Weigel, 1997; Lazard, 2002).

Africa is considered to have significant potential for aquaculture production with an estimated 34 percent of the continent considered as suitable for the farming of tilapia, catfish and carp (Kapetsky, 1994; Aguilar-Manjarrez and Nath, 1998). In spite though of various efforts since the 1950s, returns on government and international aquaculture investments appeared to be insignificant with less than 5 percent of the suitable land area being used. Sub-Saharan Africa's contribution to world aquaculture production remains at less than 1 percent (Ridler and Hishamunda, 2001; Hecht, 2006).

The FAO (1999) further reported that African capture fisheries have been fully exploited and aquaculture is not developing in SSA, whilst the demand for fish has grown. To support future needs in SSA, capture fisheries will need to be sustained and aquaculture to be developed at a rate of 8.3 percent per year up to 2020, with a current shortfall in supply of 3 million tonnes (Muir *et al*., 2005). To meet this demand, African countries currently import about 4.2 million tonnes of fishery products at a net loss of $3 thousand million. The countries of Egypt (of the Mehgred region), and Nigeria, Ghana, Zimbabwe, Malawi and Uganda in SSA are the main countries in Africa with a significant production of catfish and tilapia (Ofori *et al.,* 2010; Satia, 2011). Brummett *et al.* (2008) also emphasised the potential of sub-Saharan aquaculture to increase production. These authors outlined the factors limiting rapid growth in SSA aquaculture as poor infrastructure, volatile prices, a lack of essential inputs, political instability, poor market development, and lack of the necessary research and development to support development. In addition to these macro-economic constraints, Moehl *et al.* (2005) identified some specific constraints related to aquaculture production and commercialisation, which include lack of quality seed, unavailability of balanced feeds, limited access to technical information, poor marketing infrastructure, information and organisation, and inadequate policies and regulations. The Phuket Concensus-2010, recognizing the potential of the aquatic resources in Africa, emphasised the need for urgent development in aquaculture in SSA to accelerate social and economic development (FAO, 2012b).

1.5 Culture of Tilapia

Freshwater fishes contributes the bigger portion to global aquaculture production (56.4 percent, 33.7 million tonnes), followed by molluscs (23.6 percent, 14.2 million tonnes), crustaceans (9.6 percent, 5.7 million tonnes), diadromous (fishes that migrate between freshwater and salt water) fishes (6.0 percent, 3.6 million tonnes), marine fishes (3.1 percent, 1.8 million tonnes) and other aquatic animals (1.4 percent, 814 300 tonnes) (FAO, 2012c). Besides carps, tilapia is the second most important group of farmed freshwater and brackish water fish. It is also the most widely grown of any farmed fish. FAO (2012a and c) estimated that tilapia production in 2010 was 3.5 million tonnes valued at US$ 5.7 billion, representing 10.4 percent of farmed freshwater fish.

Based on pyramidal paintings as depicted on bas-relief from an Egyptian tomb, tilapia culture is supposed to have originated some 4000 years before the present (El-Sayed, 2006; Shelton and Popma, 2006; FAO, 2012d). According to Balarin and Hatton (1979), modern tilapia culture was first experimented in Africa in1924. It is established that the culture of tilapia exploded due to their introduction into many tropical,

subtropical and temperate regions of the world in the middle of the 20th century (Pillay, 1997; De Silva *et al.*, 2004). Since then tilapias have become one of the three most important groups of commercial fish (i.e. carps, tilapia and salmons) (El-Sayed, 2006; Shelton and Popma, 2006). Tilapias possess all the valuable characteristics desirable of a good culture fish species, such as adaptability to environments, hardiness and acceptance of wide range of feed.

The FAO (2012d) emphasised that, in the early stages of its introduction into Southeast Asia, to the uncontrolled breeding of tilapia in ponds led to excessive recruitment, reduced growth and low yield, reducing the viability of tilapia culture. However, the introduction of hormonal sex-reversal techniques in the 1970s and all-male monosex populations made possible the production of uniform, market sizes fish. Tilapia culture industry has seen a rapid expansion since the mid-1980s owing to research on nutrition and culture systems, market development and advances in processing. Available literature on tilapias (i.e. biology, culture or production and exploitation) maintained that tilapia is a native freshwater species of Africa, however, but for Egypt, Africa's contribution to global production would appear insignificant. Tilapia culture is dominated by *Oreochromis* genus, particularly *O. niloticus* and the main producers include: China (39.4 percent), Egypt (21.9 percent), Indonesia (16.9 percent), Thailand (7.1 percent) and Philippines (6.6 percent).

1.6 The Aim of the study

The genus *Oreochromis* has dominated tilapia cultivation around the world, particularly *O. niloticus, O. mossambicus* and *O. aureus*. The dominance is attributed to their resistance to considerable levels of adverse environmental and management conditions. The major drawback to tilapia culture, however, is the early female maturation at very small size (15-30g) (Mair and Little, 1991; Popma and Lovshin, 1995), and precocious breeding that usually leads to overcrowding in production systems, consequently reducing growth (Varadaraj and Pandian, 1987) which results in stunted populations. Mair and Little (1991) enumerated various methods and techniques available for the control of prolific breeding in tilapia. However, each technique or method has its own limitations. Monosex culture of all-male populations, which exhibits faster growth rates and which is usually produced through androgenous hormone sex reversal, is the preferred option, and is used extensively in the countries that produce large numbers of tilapia, e.g. China (Phelps, 2006).

Considering the problem associated with the use of androgenous hormonal treatment, such as environmental and public health concerns (Dabrowski *et al.,* 2005) and the limitations of existing methods and techniques documented by Mair and Little (1991), an alternative approach is worth investigating. Thus, there is a need to explore other technologies to control undesirable tilapia recruitment in ponds using natural reproduction inhibitors found in plants.

1.6.1 Use of plant based phytochemicals

The search for alternative methods for control of reproduction has led to the consideration of the use of medicinal plants that have been successfully used to induce sterility in laboratory animals (Bodharkar *et al.*, 1974; Das, 1980). Udoh and Kehinde (1999) succeeded in controlling the reproduction of male albino rats through the oral administration of pawpaw (*Carica papaya*) seeds. According to Shukla *et al.* (1989), an aqueous extracts of the root and bark of Moringa (*Moringa oleifera*) effectively prevented implantation in rats. These studies suggest that a contraceptive efficacy of dietary plant nutrients with antifertility or abortifacient activity exist in these medicinal plants. Pawpaw and Moringa as known medicinal plants with phytogenic (i.e. phytoestrogenic) effects, were therefore, selected to investigate their effect on sexual differentiation in and the potential to control precocious breeding in tilapia.

Stunting, poor access to current technologies (e.g. bureaucratic impediments of obtaining hormones for sex reversal), and poor management strategies have been identified as some of the major reasons for the absence and low popularity of commercial tilapia culture in sub-Saharan Africa (SSA). Pawpaw and Moringa abound in SSA and, have been demonstrated to possess abortifacient and/or antifertility properties (Das, 1980; Udoh and Kehinde, 1999; Bose, 2007).The potential of the plants can thus be exploited in the quest for a more reliable solution to control the precocious breeding of tilapia, which will contribute to encourage tilapia culture in rural SSA for poverty alleviation. The fact that both Pawpaw and Moringa seeds are available on a sustainable basis, will allow especially poor fish farmers in SSA to also make use of these techniques, should it prove to be feasible to control tilapia reproduction.

The aim of this study, therefore, is to investigate the potential and effect of dietary phytogenics to delay gonadal development in and sexual maturation of tilapia (*Oreochromis mossambicus*) to improve production performance. Phytogenic feed additives are plant-derived products or compounds that can be included in animal feeds to improve productivity of livestock, swine and poultry through the improvement of feed properties and food quality, and promotion of animal's production performance. The reported effects of phytogenic feed additives include anti-oxidative, antimicrobial, and growth-promoting effects in livestock. These effects are partially associated with improved feed consumption, which can potentially be, attributed to an improved palatability of the diet (Windisch *et al.*, 2007; Scheurmann *et al.*, 2009). According to Steiner (2009) phytogenics are a group of natural growth promoters (NGPs) or non-antibiotic growth promoters (NAGPs) derived from herbs, spices and other plants. For instance, saponins are considered as a special class of phytogenic substances, because they are able to reduce intestinal ammonia (NH_3) and hence alleviate an important stress factor to animal health (Francis *et al.*, 2002). The attempts of using phytochemicals to prevent precocious breeding in tilapia production systems are in the experimental stages (Francis *et al.*, 2002). Most of the attempts so far, used an extracted form of phytochemicals from plants, for example quercetin, genistein and diadzein (Dabrowski *et al.*, 2004; de Oca, 2005).

1.6.2 The Hypothesis

Phytochemicals known as phytoestrogens are plant-derived compounds that structurally or functionally mimic mammalian estrogens (Pelissori *et al*., 1991a; Kurzer and Xu, 1997) that control the sexual differentiation and gonadal development of fish (Nagahama, 1983; 1994). Compounds such as isoflavonoids, flavonoids and saponins, all fall in this group. Phytoestrogens (i.e. estrogenic/androgenic in nature) are found in plants such as soy, tea, fruits and vegetables (Pelissori *et al*., 1991a; 1991b; Pelissori and Sumpter, 1992; Dabrowski *et al.,* 2005), Pawpaw and Moringa (Krishna *et al.,* 2008; Kumar *et al*., 2010). The study, therefore, hypothesize that:

- Feeding fish with a diet that contains natural estrogenic/androgenic compounds will affect the gonadal activity of tilapia.
- Feeding fish with a diet (or diets) that contain natural estrogenic/androgenic compounds will affect the sexual differentiation and gonadal development of sexually undifferentiated tilapia fry and, skew the sex ratio towards a particular sex.

1.6.3 Specific Objectives

The objectives of this study were to determine the effect of the inclusion of Pawpaw and Moringa seed powder in experimental diets on the reproductive status of *O. mossambicus* during three distinct developmental stages, namely sexually undifferentiated, immature and mature tilapia. The results could serve as a basis for the use of phytogenics in feeds at different developmental stage to control reproductive behaviour in Tilapia.

The thesis is structured as a series of chapters that include:

Chapter 1 provides general introduction on aquaculture and its importance to food security

Chapter 2 presents a comprehensive literature review of tilapia culture, the problem associated with precocious breeding and methods to controlled reproduction

Chapter 3 provides a detailed presentation on the research methodology that was followed in relation to the treatment of Tilapia with Pawpaw and Moringa as feed ingredients

Chapter 4: presents results of the effect of Pawpaw and Moringa treatment on gonadal integrity of sexually mature tilapia

Chapter 5: presents results of the effect of Pawpaw and Moringa treatment on gonadal integrity of sexually immature tilapia

Chapter 6: presents results of the effect of Pawpaw and Moringa treatment on sexual differentiation of undifferentiated post hatched tilapia fry

Chapter 7 presents results of the determination of bioactive chemicals present in Pawpaw and Moringa,

Chapter 8 presents a summary of overall conclusions and recommendations

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Chapter 2

Literature Review

2.1 An Overview of Tilapia in Aquaculture

More than 70 tilapia species have been identified that originate from Africa, Jordan and Israel (Philippart and Ruwet, 1982) and occur throughout Africa, with the exception of the northern Atlas Mountains and southwest Africa (McAndrew, 2000). During the last century tilapia was introduced into various tropical, subtropical and temperate regions outside Africa for the purpose of food production, control of aquatic weeds, recreation, and research and development (El Sayed, 2006). Currently, tilapia is farmed commercially in almost 100 countries worldwide, with over 98 percent of the production occuring outside their original habitats (FAO, 2011).

Tilapia has developed into the second most important cultured freshwater fish, behind the carp. Tilapia production is growing exponentially with the global output standing at 2.5 million tonnes annually, and has therefore, been dubbed as the twenty-first century's most culturable fish (Shelton, 2002, Shelton and Popma, 2006, Fitzsimmons, 2010). Tilapia is produced and consumed on all continents, and has become the main animal protein for a majority of rural and suburban communities in developing countries, particularly, in Sub-Saharan Africa (Gupta and Acosta, 2004). This makes it more accessable in terms of the market than other successful aquaculture species such as salmon and shrimp (Norman-López and Bjørndal, 2009).

The genus *Oreochromis* is the most predominant species of tilapia being farmed globally due to certain culturable attributes including: faster growth rate, adaptibility to various environmental stress, omnivorous eating habit and reproduction in captivity. According to Rana (1997), FAO statistics indicate that the production of tilapia globally is dominated by three main species of *Oreochromis* and their hybrids, namely the Nile tilapia (*Oreochromis niloticus* Lin), the Blue tilapia (*Oreochromis aureus* Steindachner) and the Mozambique tilapia (*Oreochromis mossambicus* Peters), since the mid-1980s. The global hybrid populations of tilapia have generally, been produced from the Mozambique tilapia especially, the 'red tilapias' (Campos-Ramos *et al*., 2003). The FAO (2006) reported that the Nile tilapia (*Orechromis niloticus*) dominates tilapia production worldwide and it reached 1,703,125 metric tonnes in 2004. The percentage contribution of the different tilapia species to global tilapia production is presented in Figure 2.1 (adapted from Gupta and Acosta, 2004). Fitzsimmons (2010) attributed the rapid growth in production of tilapia in the last decades to various research and development processes, including genetic improvement programmes such as the development of the Genetic Improvement of Farmed Tilapia (GIFT) in Malaysia.

The introduction of Mozambique tilapia *(Oreochromis mossambicus)* into Asia in the 1940s and 1950s initiated the farming of tilapia. However, *O. mossambicus* farming suffered an early failure as the species had poor culturable qualities including small degraded unmarketable size and early attainment of puberty. According to Brink *et al.* (2002), the poor reputation of *O. mossambicus*, as an aquaculture species in

relation to other *Oreochromis species,* arises from the unsatisfactory culture performance of the genetically depauperate or depreciated domesticated strains, mainly in Asia. This situation has led to the species not reaching its full aquaculture potential, and it contributes only 4% (Figure 2.1) to the world tilapia production (Gupta and Acosta, 2004).

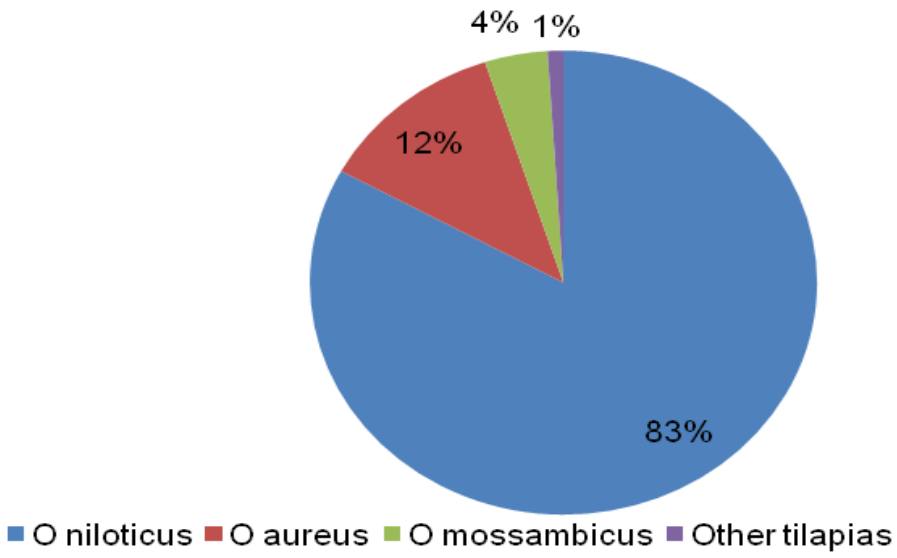


Figure 2.1 The contribution (%) of tilapia species to global production (Gupta & Acosta, 2004).

2.2 Economic and Social Relevance of Tilapia Culture

According to WorldFish Centre (2005) fish has been identified as a key vehicle or impetus for economic and social development in the United Nations Millennium Development Goals (MDGs) programme for 2015 and beyond. The WorldFish Center maintains that fisheries (i.e. capture fishery and aquaculture) have been identified as a viable option for development, which can generate wealth for developing countries, such as in Africa. They also indicate that fisheries and its products are the surest way of distributing natural wealth in African communities. Particularly to women and children in the form of nutrition and providing answers for resource governance, since women fit well in the value chain of fish distribution.

Tilapia in particular is contributing to this objective. Tilapia gained particular prominence as an international food commodity since the 1990s. Production is spread from developing countries with emphasis on food security to developed countries as a high value product. Tilapia can be cultured in many types of water bodies, including freshwater, brackishwater and marine. It is farmed in a variety of production systems such as earthen ponds, concrete ponds, pen and cages, raceways and recirculating systems. The species is further adaptable to a wide range of geographical locations and markets. Now it is being regarded as both a commodity or speciality crop such as poultry (Fitzsimmons, 2006; 2010).

According to Fitzsimmons (2010) the world tilapia production reached 3,200,000 metric tonnes (mt) in 2010, and trade increased to US$ 5,000 million (Figure 2.2). Africa and Southeast Asia are considered as the main consumers of tilapia, each consuming around 950,000 metric tonnes. It is followed by China with 500,000 mt, followed by the North America with 480,000mt, Central America 190,000, Russia Federation 66,000 and the EU 56,000 mt (Josupeit, 2010). According to Josupeit (2010) tilapia consumption in the USA has increased since 2000, becoming the 5th most sort-after fish product by consumers. In terms of fishery products found in US retail shops it is the second most popular fish behind salmon. Since 2005, the US become the highest importer of tilapia (whole frozen, fillet frozen and fillet fresh); and in 2010, importation of tilapia products reached an estimated value of US$760,000,000 (Fitzsimmons, 2010).

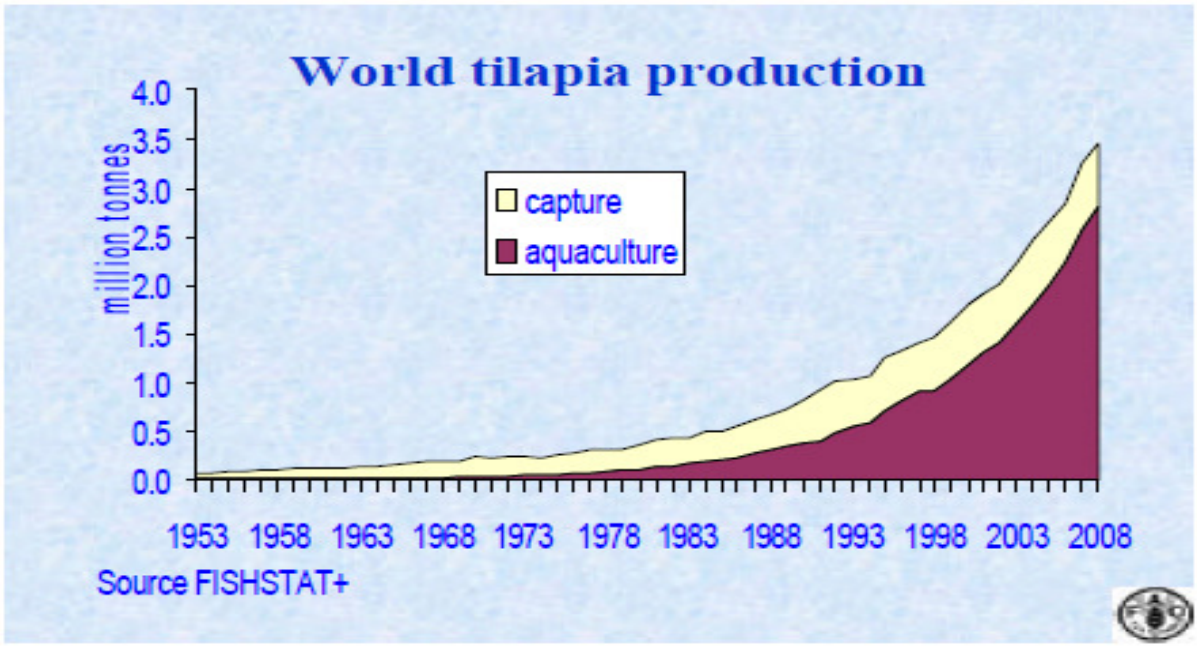


Figure 2.2 World Tilapia Production from 1953 – 2008 (adapted from Josupeit, 2010)

2.3 The Genus Tilapia

Tilapia represents a large number of predominantly freshwater finfishes belonging to the family Cichlidae. They are differentiated from other families of bony fish by the presence of an interrupted lateral line, running superior along the anterior part of the fish and inferior along the posterior portion. The name tilapia is believed to be derived from the African Bushman word meaning fish (Trewaves, 1982).

Generally, tilapias can be distinguished from other native cichlids in non-African countries by colouration and/or the presence of a pharyngeal plate used to grind vegetable matter. Another distinguishing feature is the presence of an uninterrupted lateral line, which runs superiorly along the anterior part and inferiorly along the posterior portion of the fish. This is a widespread genus, with over 3,300 species (Popma and Lovshin, 1995; Arrignon, 1998).

2.3.1 Taxonomic Classification of Tilapia

The taxonomic classification of tilapia was based on a single genus, Tilapia, until the reclassification in the early 1980s by Trewaves (1982; 1983). This was mainly due to the similarity and overlap of their morphological characteristics, and also because many species of tilapia freely hybridize in nature (El-Sayed, 2006).

According to Trewavas (1982) taxonomically, the tilapias have been identified into tree main genera on the basis of their reproductive and ecomorphological characteristics. The three genera are *Tilapia, Sarotherodon* and *Oreochromis*. They are all nest builders and substrate spawners, but differ in brooding of eggs and fry. *Tilapia* species keep and guard the developing eggs and fry in the nest; in the *Sarotherodon* the females and/or males incubate eggs and fry in the mouth; and in the *Oreochromis* species oral incubation of both eggs and fry is done only by the females.

The basic taxonomic classification of a tilapia fish (e.g. Nile tilapia) is:

Phylum:	Chordata
Subphylum:	Vertebrata
Class:	Osteichthyes
Order:	Perciformes
Family:	Cichlidae
Genus:	*Oreochromis*
Species:	*Oreochromis niloticus* Linnaeus
Common name:	Nile tilapia

2.3.2 Modes of reproduction of Tilapia

A general feature, which is not characteristic of most teleost fish, is that parental care is associated with the mode of reproduction in tilapias. According to Trewavas (1982; 1983), the genera *Tilapia, Sarotherodon* and *Oreochromis* can be distinguished on the basis of reproductive and developmental features, biogeography, and feeding and structural characteristics. Trewaves (1983) noted that within the tribe Tilapiini, parental care is said to have evolved from the ancestral substrate spawners or guarders through to oral rearing of the clutch.

The tribe Tilapiini has an elaborate breeding behavior strategy includes the laying and fertilizing of few relatively large energy-rich eggs, which are either guarded by the parents or brooded within the safety of the buccal cavity of the parents. Breeding is described as a lek (arena) system where males dig nests communally and defend territory within a spawning area. Sexual dimorphism is minimal in *Tilapia* and

Sarotherodon, but *Oreochromis* males have conspicuous breeding colours and grow more rapidly after sexual maturity (Rana, 1988; Nelson, 1995; Shelton and Popma, 2006).

The reproductive behaviour of the Genus *Tilapia, Sarotherodon* and *Oreochromis* can be described as:

Genus *Tilapia*: Includes species that are substrate spawners and do not keep the eggs in the mouth for incubation, *e.g. T. zillii*, *T rendalli*. They clean the bottom substrate and dig out the nest. During spawning, the parents secrete a sticky substance which allows the eggs to adhere to the substrate. The eggs are incubated by both parents, whom form a pair-bond throughout the spawning, incubation and early rearing of the fry. Both parents actively defend the nest, and the eggs are ventilated by their swimming actions. Once the eggs hatch, the fry remain in the vicinity of the nest and are protected by their parents for a further week until they are able to fend for themselves (Lowe-McConnell, 1958; Trewaves, 1983).

Genus *Sarotherodon*: Includes those species which are, mostly paternal mouth brooders and sometimes eggs and hatched larvae are brooded by both parents, *e.g. S. galilaeus*, *S. melanotheron*. According to Trewaves (1983) in this genus, the male and female form breeding pairs, and it is the females that initiate courtship. The nest is constructed by both fish, and in some of the species it is the male that broods the eggs and fry. Jennings and Williams (1992) reports that, whilst the male is brooding the female will aggressively defend the nest. Depending on the size of the female, clutchs produced are approximately 50 eggs in number with a diameter range between 1.5 and 4.5mm. In the black-chinned tilapia, *S. melanotheron*, it is the male that incubates the fertilized eggs in its buccal cavity (Lowe-McConnell, 1958, 1982). They are released after absorption of yolk-sac, as free-swimming fry. After about 5 days of incubation, the eggs hatch and the newly hatched embryos remain in his mouth for a little more than a week. The entire mouthbrooding behaviour or incubation within the buccal cavity ranges from 6-22 days, averaging about 2 weeks (i.e.14-18 days), depending on the species (Finucane and Rinckey 1964; Eyeson, 1979; Trewavas, 1983).

Genus *Oreochromis*: Includes those species which are exclusively maternal mouth brooders. In this group the males construct and defend a mating territory in an arena with other males in adjacent territories, and females come to find spawning partners, e.g. *O. niloticus*, *O. aureus*, *O. mossambicus*. The male builds a nest, to which he attracts a ripe female through courtship displays. The female lays her eggs in the nest and as they are fertilised, she picks them up in her mouth. Once she has laid and picked up all her eggs, she leaves the nest and moves to a quiet place to incubate. The male continues to display and attract further females to the nest, taking no more part in incubation or early rearing. During egg incubation the female cannot feed, but turns the eggs over in her mouth. Afer about 5-10 days the eggs hatch and the fry continue to develop in her mouth. Even after they absorbed their yolk sac and become free swimming, they may return to the female at any sign of danger. Eventually they outgrow their temporary shelter and are released in shallow water by the female (Trewaves, 1983; Rana, 1988; Nelson, 1995).

2.3.3 Gamatogenesis in Tilapias

The survival of animal species is dependent on their ability to pass their genetic material on to the next generation (Nagahama, 1983; Donovan and De Miguel, 2005; Mañanós *et al.*, 2009). According to Lubzens *et al.* (2010) the controlled production cycle of viable and fertile eggs into fry is a key requirement of the aquaculture industry in order to meet the ever increasing demand for fish. The reproductive cycle in fish consists of a series of developmental processes from immature germ cells to the production of mature gametes, culminating in a fertilized egg after the insemination with a spermatozoan. In both sexes, the reproductive cycles involves an initial phase of gonadal growth and development followed by maturation, ovulation or spermiation and spawning. Spawning of mature gametes into the external environment needs to be well synchronised to ensure fertilization and embryo development (Mañanós *et al.*, 2009).

The process of gametogenesis refers to the formation of the female oocyte (oogenesis) and the male spermatozoon (spermatogenesis). According to Mañanós *et al.* (2009) both male and female gametes originate from a population of embryonic primodial germ cells (PGC) that migrate during embryonic development to the place of gonad formation, the germinal epithelium. Through mitotic division, the PGC divides to form the gonia, differentiating into oogonia or spermatogonia depending on the sex of the individual. After the last mitotic division, the gonia enter meiosis and become oocytes or spermatocytes, thus completeing gamatogenesis in adult animals. Sexual reproduction in animals utilizes haploid gametes, one sex producing a relatively small number of large gametes, eggs that are rich in reserves, the other sex producing a much larger number of small gametes, spermatozoa, which evolved as highly specialized, motile genome vectors. Although the development of eggs and sperm show common principles, many aspects of gametogenesis differ between the sexes (Mañanós *et al.*, 2009; Schulz *et al.*, 2010; Evans and Ganjam, 2011; Paul-Prasanth *et al.*, 2011).

Tilapia, like other teleost fish and vertebrates, exhibit several forms of sex determination: genetic, environmental control, hermaphroditism, gonochorism (Delvin and Nagahama, 2002). Generally, tilapias are gonochorists, where individuals develop only as males or females, and remain the same sex throughout their life. Sex differentiation commences after yolk sac absorption and initiation of exogenous feeding (Yamazaki, 1983; Nakamura *et al.*, 1998).

2.3.4 Oogenesis

According to Mañanós *et al.* (2009), the process of oocyte development (Figure 2.3) in female teleost fish starts with the mitotic proliferation of the oogonia that becomes a primary oocyte when entering meiosis. The primary oocyte then undergoes a secondary growth stage known as vitellogenesis. It is a process that involves the synthesis, incorporation of vitellogenin (VTG), and the filling of the cytoplasm with yolk that will serve as food for the developing embryo. The final phase of oocyte development involves maturation, where the nucleus moves to the animal pole, and the oocyte is hydrated. Oocytes are moved, through final stages of ovulation, into the ovarian or abdominal cavity from where they are released into the water during spawning.

The tilapia is an oviparous species, where the female fish produce yolk-containing eggs. It is an asynchronous ovulator/spawner, in which eggs are recruited from a heterogenous population of developing oocytes, which are then subsequently ovulated in several batches during each spawning season (Rana, 1988). Ovaries in the adult fish contain oocytes at various stages of development, granulosa cells, theca cells (Morrison *et al.*, 2006; Lubzens *et al.*, 2010).

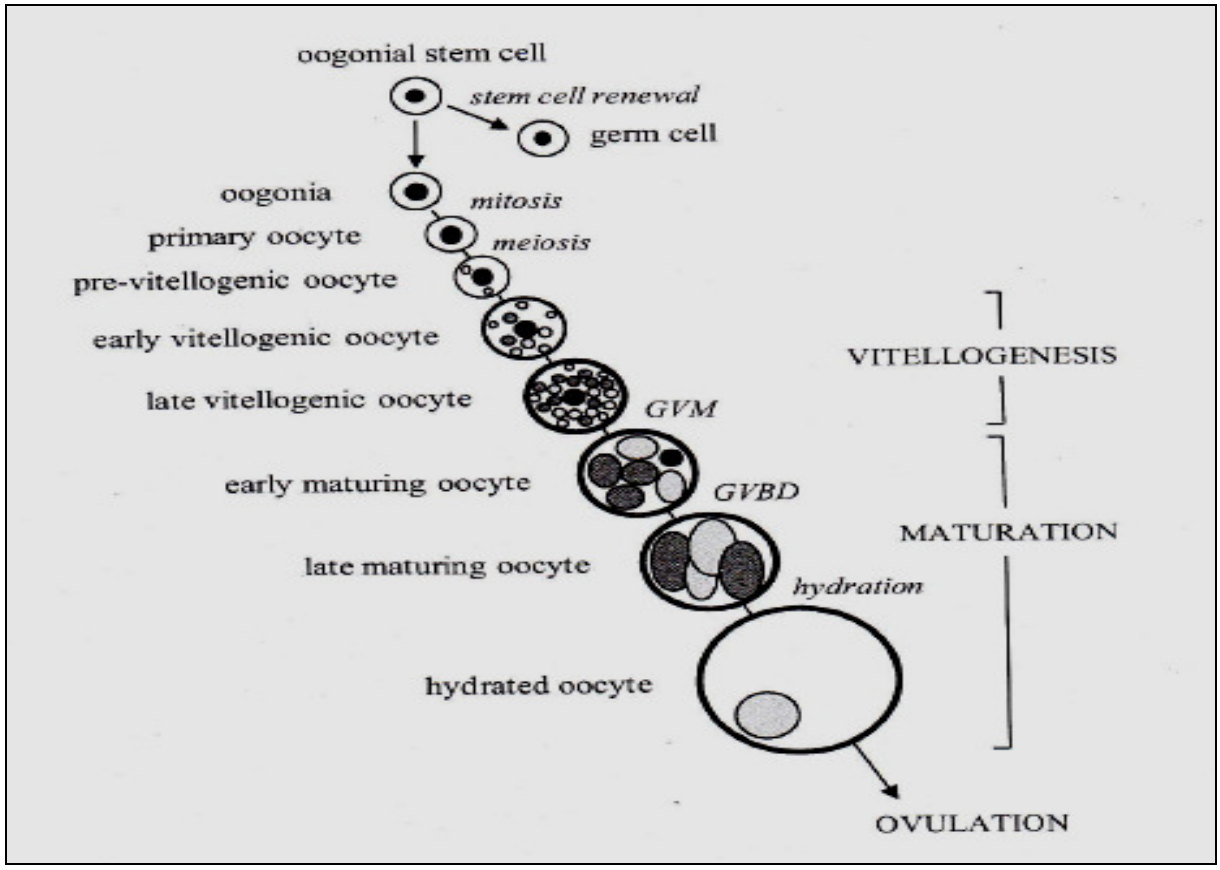


Figure 2.3 The process of oocyte development and maturation in female fish (Adapted from Mañanós *et al.*, 2009)

2.3.5 Spermatogenesis

Spermatogenesis is the process that occurs within the testes producing spermatozoa or sperm. It is a lengthy process, which occurs within seminiferous tubules of the testis with the cyclical division of spermatogonian stem cells through mitosis, to maintain their own chromosome numbers, to produce primary spermatocytes. The spermatocytes then undergo meiosis to produce haploid spermatids, which differentiate without further division into spermatozoa. Spermatogenesis thus occurs in three phases: meiosis, spermiogenesis and spermiation (Johnson *et al.*, 2000; Patiño and Redding, 2000).

In all vertebrates, from fish to mammals, the testis is composed of two main compartments, the intertubular (or interstitial) and the tubular compartment. The intertubular compartment contains steroidogenic Leydig cells, blood/lymphatic vessels, macrophages and mast cells, neural and connective tissue cells, the latter

being continuous with the tunica albuginea i.e. the testis organ wall. The tubular compartment is delineated by a basement membrane and peritubular myoid cells and houses the germinal epithelium. This epithelium contains only two cell types, the somatic Sertoli cells and the germ cells, which are found at different stages of development (Mañanós *et al.,* 2009; Schulz *et al*., 2010). Serttoli cells support and regulate the development of spermatocytes (Patiño and Redding, 2000).

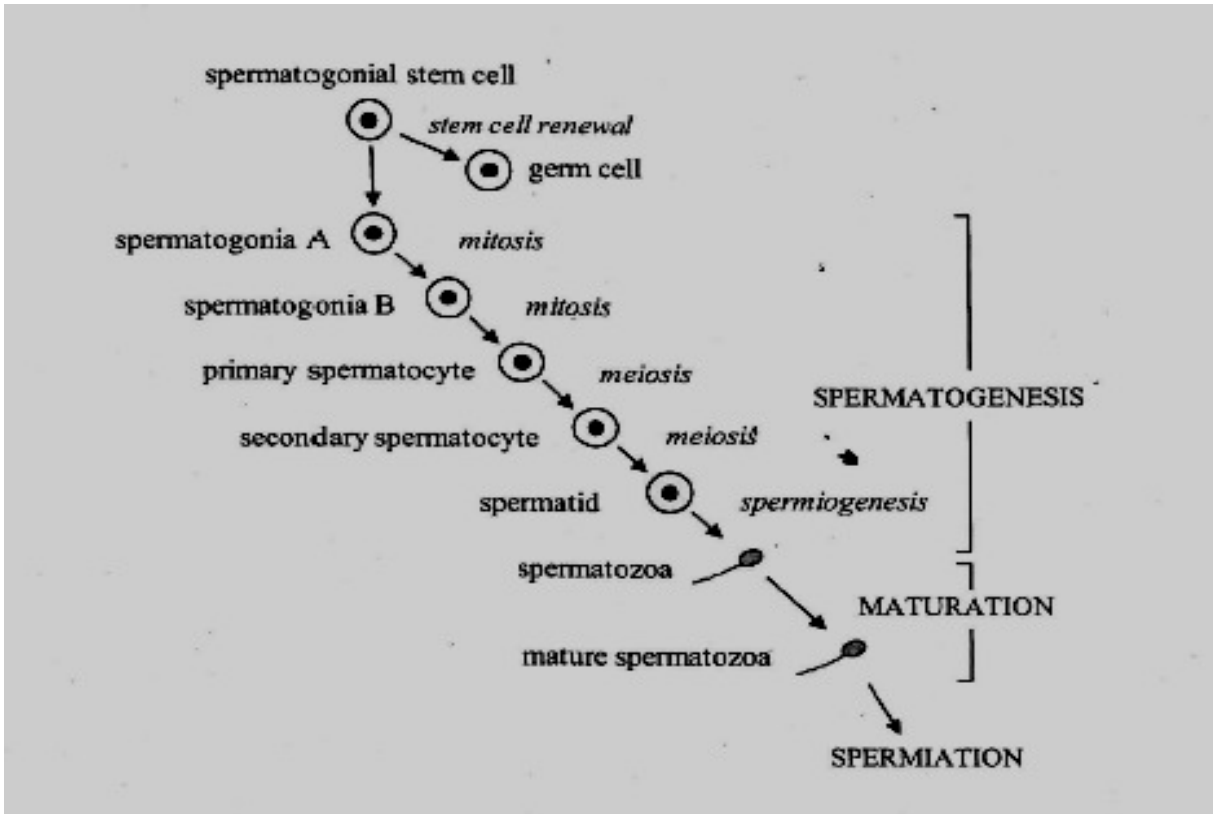


Figure 2.4 The process of spermatozoa development and maturation in male fish (Adapted from Mañanós *et al*., 2009)

Spermatogenesis in males commences with the mitotic multiplying of spermatogonia into spermatocytes (Figure 2.4), which mature to become spermatids. Unlike meiosis in oocytes, meiosis in spermatocytes is a continuous uninterrupted process (Patiño and Redding, 2000). During spermiogenesis, the spermatids differentiate into flagellated spermatozoa, a process that involves a size reduction, and flagellum are formed before release into the testicular lumen. The flagellated spermatozoa in the testicular lumen undergo the process of maturation to acquire the ability to fertilize. The mature spermatozoa are stored in the collecting duct of the testes until they are released into the water during synchronized spawning.

2.3.6 Endocrine Control of Reproduction

The hypophyseal-gonadal axis (HGA) / brain-pituitary-gonad axis (BPG) are involved in the control of reproduction in fish. The hypophysial and gonadal hormones are intimately involved in the regulation of the

timing of reproduction, which is affected by environmental conditions. It is well established that gonadotropins are the primary hormones that regulate the process of gametogenesis. Gonadotropin (GTH) produced by gonadotropin cells are most closely associated with reproduction, stimulating the synthesis of gonad steroid hormones, uptake of vitellogenin, oocyte maturation and ovulation, and spermiation (Nagahama, 1994; Mousa and Mousa, 1999; Strüssman and Nakamura, 2002; Mañanós *et al.*, 2009).

In the process of sexual maturation and gonad development in tilapia, the most vital role is played by the endocrine hormones like vitellogenin, 17β-oestradiol, testosterone, and 11-ketotestosterone. Development of secondary sexual characteristics such as bright and shiny body colour, bluish fish with reddish margins, prominent urogenital papillae and extended anal and dorsal fins are also controlled by these sex steroid hormones.

Normally in female fish, egg yolk is derived from a precursor of a lipophosphoprotein-calcium complex called vitellogenin, which is synthesized by the liver, released into the blood and finally sequestered by the oocytes by means of a receptor-mediated endocytotic process (Tyler *et al.*, 1987; Tyler and Sumpter, 1996). The yolk precursor is believed to be synthesized during the vitellogenesis phase of ovarian growth under the influence of oestrogenic control (i.e. vitellogenin production is normally stimulated by 17β-oestradiol hormone). On the other hand, testosterone acts as a precursor in oestrogen synthesis. Ultimately, vitellogenin and all other sex steroid hormones have interlinked functions to regulate oocyte maturation. Similarly in male fish, sex steroid hormones also regulate the development and maturation of sperm (Nagahama, 1983).

According to Mañanós *et al.* (2009) an individual's ability to produce and release mature viable gametes is dependant on the proper functioning of all the components of the reproductive processes from gametogenesis to spawning. A successful spawning involves a synchronized secretion of pituitary and gonadal hormones throughout the reproductive cycle. During culture, various factors such as handling stress or the absence of environmental stimuli can affect the brain-inhibiting neuroendocrine secretions that could block the reproductive axis and inhibit reproductive success. Therefore, any form of stress that can interfere or interact with the functioning of the BPG axis, can affect or prevent reproductive activity, thereby allowing the fish to use reserve energy for somatic growth instead of gonadal development.

2.3.7 Puberty control of farmed fish

Attaining early puberty is one of the major problems in farmed fish, such as tilapia (Mair and Little, 1991). Puberty is, regarded as the developmental period covering the transition from an immature juvenile to a mature adult reproductive system. The core components of the reproductive system are the gonads and the endocrine system that regulate the gonadal activities, the brain–pituitary–gonad (BPG) axis (Schulz and Goos, 1999; Okuzawa, 2002; Mañanós *et al.*, 2009).

The BPG axis, also known as the reproductive axis involves the pituitary gonadotropins (GTHs), controlled by Gonadotropin-Releasing Hormone (GnRH), Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH), key factors in the endocrine control of reproduction. According to Mañanós *et al.* (2009) the success of

reproductive maturation and viable gamete release depends on the correct functioning of all components of the reproductive axis throughout the entire cycle, from gametogenesis to spawning. The pituitary gland is stimulated by GnRH to secrete FSH and LH into the bloodstream to act on the gonad, increasing the synthesis of gonad steroid hormones, which are the ultimate effectors of gonadal development. The stress associated with captivity or absence of appropriate environmental conditions in culture facilities may act on the brain-inhibiting neuroendocrine secretions, and thus blocking the reproductive axis, which could inhibit reproductive success (Mañanós *et al*., 2009; Pavlov *et al*., 2009).

In the teleost fish, puberty commences after gonadal sex differentiation characterized by the onset of spermatogenesis in males and vitellogenic ovarian development in females (Okuzawa, 2002; Patiño and Sullivan, 2002). It is, established that the steroid estrogen is produced before and after ovarian differentiation (Nagahama, 1983), thus inhibition of estrogen synthesis could increase the proportion of males. Pifferrer (2001) reported that sex steroids and their actions are involved in the process of sex differentiation in fish. The author maintained that, during sex differentiation sex steroids act mainly as morphogenic factors. However, later in the life cycle, during sexual maturation, sex steroid act mainly as activational factors.

In sexually mature fish, physiological regulation is mediated through the hypothalamus-pituitary (hypophysis)-gonad (HPD) axis that integrates information from the external environment (e.g., photo period, water temperature), social stimuli from potential mating partners (e.g. courtship) and internal signals (e.g. nutritional status) to regulate the consecutive processes of gonad maturation, spermatogenesis or oogenesis, reproductive behavior and spawning (Segner, 2011).

The basic features of the reproductive endocrine system are similar among teleost fish – despite their wide spectrum of reproductive strategies and tactics of breeding (Mañanós *et al*., 2009; Pavlov *et al*., 2009). The timing of onset of puberty in fish generally, is determined by several external and internal factors including photoperiod, water temperature, food availability, somatic growth and gonadal status, all of which could be manipulated under farm conditions. For instance, photoperiod has been manipulated to delay the initiation of puberty in salmonids (Taranger *et al*., 2010). Both endogenous and exogenous steroid hormones are considered to have positive effect on pubertal development. Schulz and Goos (1999) proposed that sex steroids are the natural inducers of onset of puberty in male African catfish. In juvenile male African catfish, long-term treatment of 11-ketotestosterone or its precursor stimulated testicular growth and spermatogenesis, probably through direct effect on the testes (Cavaco *et al*., 1998; Schulz and Goos, 1999). Okuzawa (2002) stated that a combination of water temperature and photoperiod manipulation can be effectively used to determine the timing of puberty, but the participation of sex steroids in the onset of puberty has not been determined.

The main characteristic of puberty is the accompanying activation of the two main functions of gonad i) the production of germ cells, and ii) the bio-synthesis of reproductive hormone (Taranger *et al*., 2009). Sex hormones are required in the various stages of germ cell development in both males (androgens and

spermatogenesis) and females (estrogens and vitellogenesis). According to Okuzawa (2002) the endpoint of puberty is considered as the time point that coinsides with the successful production of first batch of fertile gametes (spermiation and sperm hydration in males and ovulation in females).

2.4 Tilapia Culture

Tilapia possesses most of the desirable attributes of a species suited for culture activities (Liao and Chen, 1983; Teichert-Coddington *et al*., 1997; Lovshin, 2000; Muir *et al.,* 2000; Mair, 2001; El-Seyed, 2006), and can be considered an ideal candidate for aquaculture in developing countries, especially sub-Saharan Africa (SSA). Desirable attributes include traits such as:

a. Hardy and grow quickly reaching the usual market size of 200-400g in six months
b. Tolerance to a wide range of environmental conditions (such as temperature, salinity, low dissolved oxygen, high density, and high ammonia)
c. Resistance to stress and disease
d. Reproduce readily in captivity and short generation time.
e. Feeding on low trophic levels, with very versatile food habits and acceptance of artificial feeds immediately after yolk-sac absorption
f. Adapt to a variety of culture conditions (ie low density pond to very intensive systems, mono to polyculture)
g. Considered as palatable, marketable, and nutritious product

2.4.1 Tilapia Farming Systems

In tilapia farming, the grow-out strategies involve simple to very complex techniques. The charateristics of simple strategies include little control over water quality and nutritional value of the food supply with a low fish yield. Imposition of greater control over water quality and fish nutrition results in increased cost and fish yield per unit area. The choice of any farming system depends largely on natural resources, infrastructure, management skill, availability of capital, cost and availability of nutrient inputs and market value of the fish (Popma and Lovshin, 1995; El-Sayed, 2006).

Extensive Farming Systems

In an extensive system, the culture unit is usually a drainable earthen pond with very little control of the water supply. Stocking density is very low, 1,000-2,000/ha, and mostly, nutrition is from natural food organisms produced by the nutrients contained in the pond soils and water. It has a low yield ranging from 300 to 700 kg/ha/crop. This farming system is economically viable because land is cheap and pond construction costs are incorporated into other usage such as irrigation or cattle watering. Extensive systems, also described as smallholder pond systems, usually form part of rural aquaculture development. The ponds are fertilized with manure or agricultural waste to stimulate plankton growth on which the fish feed.

Semi-Intensive Farming Systems

Semi-intensive systems are considered to be the production of tilapia in fertilized ponds, with low quality supplemental feeding (Arrignon, 1998; El-Sayed, 2006). Semi-intensive systems are directed to produce low-cost tilapia via low production inputs, with the ability to contribute to food security in rural areas of developing countries of sub-saharan Africa. Popma and Lovshin (1995) explained that this level of intensity in management is common for small-scale tilapia operations with limited capital or where high quality feeds are unavailable. The grow-out system is often an earthen pond that is enriched with inorganic and /or organic fertilizers to increase natural productivity. Supplemental feed to boost nutrition usually comes from agricultural by-products and are used to increase yields. Stocking rates of tilapia are 5,000 to 20,000/. The fish yields are often high with 1,500 to 2,500 kg/ha/crop in ponds fertilized with chemical fertilizers, 2,000 to 6,000 kg/ha/crop in manured ponds and 4,000 to 8,000 kg/ha/crop in fertilized ponds with supplemental feed from agricultural by-products. The typical production cycle is six months.

Intensive Farming Systems

In this type the culture unit is mainly earthen ponds with a controlled water supply and discharge, supplemented with routine and/or continuous aeration. Stocking density is 10,000 to 30,000/ha, where the fish is fed a high quality pelleted diet that provides all the nutritional needs of the fish. Daily feeding is a ration of 2 to 4 % of total fish biomass, where the maximum daily rates is commonly set at 80 to 120 kg/ha. Aeration is routinely provided to maintain dissolved oxygen concentrations within a desirable range. One of the major aims of intensive culture is the maintenance of high stocking densities in order to maximize the production with the minimal use of water, depending exclusively on artificial feed and water reuse and /or exchange (El- Sayed, 2006).

Other Production Systems

The ability of Tilapia to tolerate high densities, a wide range of environmental conditions, in addition to high resistance to stress, disease, and handling, has led to the development of a variety of intensive production systems such as tanks, cages & pens, raceways, recirculatory and aquaponic systems. According to El-Sayed (2006), in these super intensive systems, water quality (temperature, dissolved oxygen, salinity, ammonia, pH, dissolved solid metabolites), nutrition and feeding, and stocking densities, are the key ingredients that determine the success or failures of production. Tilapia can also be cultured in polyculture system with carps or catfish in and / or integrated system with other livestock such as poultry.

2.4.2 Tilapia growth and development

Sexual maturity in tilapia species is a function of age, size, and environmental conditions (Rana, 1988; Shelton and Popma, 2006) followed by successive reproductive cycles at intervals of 4 to 6 weeks (Coward and Bromage, 2000). One of the main features of tilapia reproduction is the plasticity of initial sexual maturation relative to size and age. Even in captivity, tilapias complete maturation and spawn without interference, and the ease by which they breed is one of the reasons why tilapia farming has spread

throughout the world. However, their rapid reproduction can cause problems associated with overpopulation and reduced growth (Arrignon, 1998; El-Sayed, 2006).

Tilapia mature at a later age and larger size under natural conditions compared to those raised in culture ponds. For instance, it is, established that in several natural lakes in East Africa, *Oreochromis niloticus* matures at about 10 to 12 months at a size of 350 to 500g. The same population in farm or culture ponds, under conditions of near maximum growth, will reach sexual maturity at an age of 5 to 6 months and 150 to 200g (Popma and Lovshin, 1995). When growth is slow in farm ponds, sexual maturity may be delayed by a month or two, but fish may still spawn at weights as low as 20g (Mair and Little 1991). Under fast growing conditions in culture ponds *O. mossambicus* may reach sexual maturity in as little as three months of age, at which time they rarely exceed 60 to 100g. In poorly fertilized ponds, sexually mature fish may be as small as 15g. Female *O. mossambicus* reach 50%-sexual-maturity at 109 mm standard length (SL), while males matured at 105 mm SL (Popma and Lovshin, 1995; Popma and Shelton, 2006).

Farmed tilapias often reach sexual maturity before they are large enough to be harvested. This results in uncontrolled reproduction and subsequent reduction in growth rate. Hepher and Pruginin (1982) observed that tilapias reach sexual maturity in 3 to 6 months, and spawn before they reach a marketable size. Thus, sexual maturity can occur at very small sizes, as low as 15-20g (Mair and Little, 1991). The *Oreochromis* species are gonochoristic and mature in the first year at the earliest opportunity and reproduce in almost any conditions. Generally, *O mossambicus* reaches sexual maturity at a much smaller size and younger age than both *O niloticus* and *O. aureus* (Rana, 1988; Shelton and Popma, 2006). In aquaria it can mature and reproduce at 45mm and 4g (Noakes and Balon, 1982).

In any animal production system, good nutrition is considered as one of the most essential components to produce a healthy, high quality product and, maximise yield and profit. In tilapia culture, nutrition is critical since feed takes up about 40-70% of production costs. The efficiency of food utilisation is high in farmed fish, because feed quality and quantity and the timing of its distribution are optimised to meet metabolic requirements. One of the major problems in tilapia culture, is the tendency of females to mature and reproduce at small sizes. Rearing both sexes of tilapia in the same pond results in overpopulation. This high reproductive efficiency is related to early sexual maturation, prolific spawning, and fry care. This early maturation poses a potential problem for culture, because energy for somatic growth is, diverted into gonad development and reproduction (territorial/courtship behaviour and the metabolic cost of gamete production). Furthermore, the fry and fingerlings compete with stocked fish for available space and food resources, thereby hindering the growth of stocked fish within the culture system, where space and food rapidly becomes limited. Competition for supplementary feed and natural pond food between the stocked tilapia and their young reduces growth rates and results in stunted populations (Mair and Little, 1991; Toguyeni *et al.*, 1997).

Energy requirements of fish are increased during the maturation of the gonads and by the demands of spawning activity. This increasing demand for energy is caused by energy use during the formation of

gonadal products. During the later stages of exogenous vitellogenesis, large amounts of yolk material are absorbed by the oocytes in the ovaries of the female taking fat and protein from external (dietary) and internal (somatic) sources. Despite this increase in nutritional requirements, many fish cease to feed during the period of gonadal maturation that immediately precedes spawning. Therefore many fish must depend on endogenous nutrients at a time when considerable energy is required for spawning activity and, in females, final ovarian maturation. This consequently leads to a loss in somatic weight, retarded growth, decline in condition and depletion of energy reserves of many fish during the gonadal maturation.

Consequently, faster growth rates and a delayed age at maturation are two important breeding goals. Body weight and age at maturity are two related and important traits for economical fish production. Age at first maturity is an economic characteristic for farming of aquatic organisms. Early maturation and precocious breeding, as observed in species such as tilapias, salmonids, and common carp, have been found to retard growth rate for several months, affecting flesh quality, and often causes increased mortality. In this way, the ability to delay or suppress puberty would be beneficial (Nevdal, 1983; Varadaraj and Pandian, 1987; Crandle and Gall, 1993; Okuzawa, 2002).

Tilapia growth is influenced by genetics as well as environmental factors such as the quantity and quality of feed, water quality, water temperature, sex, age, size, health and stocking density. In tilapia, males grow much faster than females (Pullin, 1984) and hence, all-male populations are generally the preferred choice for aquaculture (Das *et al.*, 2012). It is established that the fastest growers of tilapia strains include males of pure strains of *O. niloticus* and hybrids with *O. niloticus* as a parent, especially *O. niloticus* x *O. aureus* hybrids. *Oreochromis mossambicus* males grow more slowly, especially when larger than 200 to 300g. Male *O. niloticus* stocked at 20-30 g of size and provided with adequate nutrition and high water quality with optimal water temperature, can reach a size of 450-500g in 150 days; subsequent growth to 800-1000g may be 4 to 5 g/d with good feed and water quality. Generally, *O. niloticus* cultured under ideal conditions will grow from 1g fry to 800g in 1 year (Popma and Lovshin, 1995). Somatic growth in fish is believed to be controlled by three families of hormones, i.e. growth hormone, thyroid hormone and sex hormones (Sumpter *et al.*, 1996). In tilapias, growth is influenced by androgens, food availability, hierarchical dominance and sexual dimorphism where males grow larger and faster than females (Fauconneau *et al.*, 1997; Toguyeni *et al.,* 2002).

2.4.3 Environmental factors sffecting growth and development

Water quality management is a key factor for successful aquaculture practice. The major water quality parameters and their interrelationships affect fish growth, health and reproduction (El-Sayed, 2006). These environmental parameters include temperature, dissolved oxygen, salinity, ammonia and nitrite, pH, photoperiod and water turbidity.

Temperature is one of the cardinal factors that affect the physiology, growth, reproduction and metabolism of tilapias (El-Sayed, 2006). Tilapias are thermophilic fishes and are known to tolerate a wide range of water

temperatures (Philipart and Ruwet, 1982). The optimal temperature range for normal growth and reproduction is between 25-30^{o}C. The lower temperature limit below which feeding in tilapia is reduced is 20^{o}C. It inhibits feeding at 16^{o}C, while severe mortality occurs at 12^{o}C. The upper limit which most tilapias cannot tolerate is above 40-42^{o}C (Chervinski, 1982; Philipart and Ruwet, 1982).

Dissolved oxygen (DO) is one of the limiting environmental factors that affect fish feeding, growth and metabolism. The concentration and dissolvement of oxygen in a water body is affected by photosynthesis, respiration and diel fluctuation. Most tilapia species can survive dissolved oxygen (DO) concentrations less than 0.5 mg/L of routine dawn level, which is considerably below the tolerance levels for most other cultured fish. Tilapia is able to survive in water with low DO is due in part to their ability to extract dissolved oxygen from the film of water at the water-air interface when DO is below 1 mg/l (Philipart and Ruwet, 1982). In spite of tilapia's ability to survive acute low DO, El-Sayed (2006) emphasised that ponds should be managed to maintain DO level above 2 or 3 mg/l as metabolism and growth are depressed when levels are below this level for prolonged periods.

All the commercial valued tilapias are freshwater species, however, they are all tolerant to brackish water. *Oreochromis niloticus* is the least saline tolerant of the commercially important species, but can grow well at salinities up to 15 ppt, whilst, *O. aureus* grow well in brackish water and up to 20 ppt salinity. It is repoted that *Oreochromis mossambicus* and *O. spirulus* can grow and spawn in full strength seawater, while *O. aureus* and *O. niloticus* can reproduce in salinities of 10 to 15 g/L (Philipart and Ruwet, 1982; El-Sayed, 2006). Owing to this level of salinity tolerance, *O. spilurus, O. mossambicus* and the red tilapia hybrid derived from *O. mossambicu* are usually the preferred choice for culture in salt water (Philipart and Ruwet, 1982; Popma and Lovshin, 1995).

The best water for tilapia growth is near neutral or slightly alkaline, with the lethal alkaline limit is pH 11 and above (Philipart and Ruwet, 1982). The commonly cultured tilapia species survive at pH as low as 5, but growth is reduced in acidic waters, probably due to less production of natural food organisms. Phiipart and Ruwet (1982) contend that an increase in pH upto 10 does not seriously affect tilapia production.

Most of the nitrogenous waste from fish is in the form of un-ionized ammonia ($UINH_3$), which is toxic to the fish and ionized NH_4^+, which is non-toxic. The toxicity of ammonia is closely correlated with DO, CO_2 and pH and, to a lesser extent, by water temperature (Chervinski, 1982). According to El-Sayed (2006), the toxic level of un-ionised NH_3 that affects the growth of Nile tilapia is in the range of 0.07 and 0.14 $UINH_3$/L. Ammonia does not very much tilapia affect when DO level is high (7 – 10 mg/L).

Photoperiod has been found to have effect on daily rhythmic activity, promote fish growth, metabolic rates, body pigmentation, sexual maturation and reproduction (Biswas *et al*., 2005; El-Sayed, 2006). Ridha and Cruz (2002) reported that photoperiod has an effect on gonad development, fecundity and spawning frequencies in the Nile tilapia.

Water turbidity can be a major concern in pond systems, where the water is fertilized. Fish growth, feed efficiency and survival can be affected with increasing water turbidity, particularly in fry and young fry as it can clog their gills (El-Sayed, 2006).

2.5 Reproductive aspects of Tilapia culture

Gender control is of importance for maximizing the economic efficiency of commercial culture production (Donaldson, 1996; Okuzawa, 2002). Ideally, fish in aquacultural should not reproduce in the culture system before reaching marketable size (Phelps, 2006). In many species one sex grows faster, matures later or has a higher market value than the other sex, thus in tilapias, males are preferred (Donaldson, 1996). The farming of monosex populations is, reported as solutions to the problem of early sexual maturation and unwanted reproduction. According to Beardmore *et al.* (2001), the culture of monosex fish seeks to achieve the following; reduction of sexual/territorial behaviour, elimination of reproduction, achieving a higher average growth rate, reduction of variation in harvest size and reduction of risk of environmental impact resulting from escapes of exotic species.

Tilapias have numerous advantages as an aquaculture species (Teichert-Coddington *et.al*, 1997). In populations of tilapia, males grow faster and are more uniform in size than females. Monosex male tilapias have many characteristics that have made them excellent aquacultural candidate. Some of the major attributes include: controlling of over- reproduction; high growth rates and feed utilization efficiency; higher energy conservation; reduced aggressiveness; greater uniformity of size at harvest; better fresh quality and appearance and most of all high tolerance to severe environmental conditions, such as temperature, dissolved oxygen, low salinity and low ammonia (Lovshin *et al*., 1990; El-Sayed, 2006). The *Oreochromis* species of the tribe Tilapiini dominates tilapia farming and, these species have been found to have a low relative fecundity, about 6,000 to 13,000 eggs per kilogram per spawn (Rana, 1988). This low fecundity is compensated by high survival rate and frequent spawning, both in the wild and aquaculture environment. Females use considerable energy in egg production and do not eat when they are incubating eggs (Rakocy and McGinty, 1989). From this perspective, tilapia farming is very challenging to farmers. The paradox involving tilapia reproduction in aquacultural setting has resulted in the need to develop various techniques to control unwanted reproduction (Phelps, 2006).

2.5.1 Control of Reproduction in Farmed Tilapias

Early maturation and the precocious breeding pattern of tilapias have prompted the development of several methods to control reproduction in order to produce good marketable sized fish. Mair and Little (1991) referred to these methods as intermittent harvest by removing parent fish; manual sexing before sexual maturation; stocking with predators/piscivorous fish in growout ponds; stocking of fish at high densities; stocking of tilapia in net cages in mesh size greater than 2.5 cm; growing in salinity higher than 25ppt for some tilapia species; sterilization through application of heat, cold or pressure shocks to fertilized eggs and hybridization, particularly of crossing between the *Oreochromis* species. The use of feed rationing has also been considered, however, the usage as an agent of change in the physiology of fishes have been directed

towards gonadal development and spawning (Gunasekera, 1995; Rinchard, 2002; Celik and Altun, 2009) and growth (Santiago, 1987; Ron *et al*., 1995; Al-Hafedh, 1999; Rinchard, 2002; Celik and Altun, 2009).

2.5.2 Methods of Producing Monosex Tilapia Populations

The most common methods as practiced are: manual sexing / hand sorting, hybridization, hormonal sex-reversal and genetic manipulation (Pandian and Varadaraj, 1987; Guerrero, 1988; Mair and Little, 1991; Popma and Lovshin, 1996; Mair *et al*., 1997).

Manual-sexing/Hand sorting of Sexes

This method is based on the sexual dimorphism observed in the urogenital papilla that allows for the separation of males from females by visual inspection of the external urogenital pores, often with the aid of dye (e.g. gentian blue) applied to the external papillae. The genital papilla of male is larger than that of the females and has two openings; the urogenital opening, where the milt and urine are excreted and the anus, for the discharge of fecal waste. The female has a flatter and shorter papilla with three openings; the anus, the urethra (for excretion of urine) and the oviduct, where eggs pass through. Secondary sex characteristics may also be used to help distinguish sex; for example, the differences in the dorsal and anal fins which are pointed in males but rounded in the female (Chervinski and Rothbard, 1982).

Manual sexing technique requires rearing a population of fry for two to three months to fingerlings size, followed by the elimination of all females. *Oreochromis mossambicus* fingerlings can be separated at 15-25g when the papilla is quite discernible. *O. niloticus* and *O. aureus* fingerlings are more difficult to separate from females by the genital papilla, compared to *O. mossambicus*. Accuracy of sexing depends on the skill of the workers, the species to be sorted and its size. Experienced workers can reliably sex 15g fingerlings *O. mossambicus,* 30g of O*. niloticus* and 50g of O*. aureus,* achieving accuracies up to 95 percent (Rakocy and McGinty, 1989; Popma and Lovshin, 1996). Sexing accuracy increases with increasing size of the fish (El-Sayed, 2006), fish heavier than 50g can easily be sexed. The presence of a few remaining females can inadvertently derail all the effort involved in sexing (Pandian and Varadaraj, 1987).

Hand sorting is one of the main methods used in Sub-Saharan Africa where other techniques are unavailable or difficult to access. Although manual sexing is simple, it is expensive in terms of time and labour, requires qualified personnel and usually results in 3 to 10 percent of error. It has been identified that, manual sexing leads to the under utilization of farming infrastructures, interrupts production, reduces feed efficiency and lowers productivity.

Hybridization

Hybridisation of two species may result in monosex populations, and the phenomenon of producing all-male or nearly all-male hybrids has been observed in fish. The best known, examples are in the tilapias, particularly, the *Oreochromis* species. The research on production of monosex tilapia hybrids was triggered by Hickling's 'Malacca Tilapia Hybrids' (Hickling, 1960). These resulted from a cross between females of the

Malacca strain of *O. mossambicus* with male *O. hornorum*, from Zanzibar. The process of hybridization to produce monosex tilapia population involves the selection of crossing a homogametic male tilapia of one species with a homogametic female of another, resulting in all-male hybrids (Hickling, 1960; Prugnin *et al.*, 1975; Lovshin, 1982).

Since the pioneering work of Hickling (1960), that showed that tilapia hybrids have the potential of controlling reproduction in grow-out ponds several crosses have been identified among mouthbrooding species of tilapia that results in all-male or nearly all-male hybrids (Prugnin *et al.*, 1975; Wohlfarth, 1994; Beardmore, 2001) (Table 2.1).

Table 2.1 Hybridization of tilapia species to produce all-male populations (adapted from El-Sayed, 2006)

Male	Female	% Male	Reference
O. aureus	*O. niloticus (Ugandan strain)*	96-100	Prugnin *et al.* (1975)
O. aureus	*O. niloticus (Stirling Strain)*	100	Marengoni *et al.* (1998)
O. aureus	*O. mossambicus*	100	Beardmore *et al.* (2001)
O. hornorum	*O. mossambicus*	100	Hickling (1960)
O. hornorum	*O. spilurus*	100	Prugnin (1967)
O. hornorum	*O. niloticus*	100	Wohlfarth *et al.* (1994)
O. macrochir	*O. mossambicus*	100	Majumdar *et al.* (1983)
O. maccrochi	*O. niloticus*	100	Prugnin (1967)

Hybridization was largely ineffective in controlling unwanted reproduction due to the complexity of maintaining pure parental lines and species. Commercially available red tilapia strains are mostly hybrids and products of cross breeding involving as many as four different species in which *O. mossambicus* and *O. niloticus* are predominant (McAndrew *et al.*, 1988). The Taiwanese red tilapia has been reported as a hybrid between albino *O. mossambicus* and *O. niloticus* (Kuo, 1969, 1988; Liao and Chang, 1983).

Although interspecific and intergeneric hybridizations have been used to produce various forms of all-male tilapia, Varadaraj and Pandian (1989) maintained that the technique has limited scope due to difficulty in maintaining pure parental stocks that can consistently produce 100% male offspring. In addition the poor spawning success and incompatibility of breeds resulting in low fertility.

Hormone-induced sex reversal

Limitation of the previous methods has led to investigations in the use of hormonal sex reversal to produce monosex tilapia populations. Generally, the hormones are incorporated into larval feeds and administered to undifferentiated larvae at very early larval stage, usually at first exogenous feeding, for sufficient time to enable sex-reversal to be effective. The hormonal methods involve the addition of steroids in feeds for a short period during the fry stage with easy of application and relatively in consonance with the production of nearly all male populations. Hormonal sex-reversal has since been used extensively to produce monosex fish for aquacultural purposes.

The idea to use hormones for production of tilapia monosex population emanated from pioneering work by Yamamoto in the 1950s on functional sex reversal with the medaka, *Oryzias latipes.* He demonstrated that androgen-induced masculinisation, whilst estrogen resulted in feminization (Phelps, 2006). The technique has been used successfully to change the sex ratio of several teleost fishes, such as rainbow trout, *Oncorhynchus mykis* (Yamazaki, 1976); grass carp, *Ctenopharyngodon idella* (Stanley, 1976); and tilapia (Nakamura and Takahashi, 1973; Jalabert, *et al*., 1974; Guerrero, 1975; Tayamen and Shelton, 1978; Jensen and Shelton, 1979; Hopkins *et al*., 1979).

This technique consists in masculinisng the entire population of fry by the incorporation of a steroid hormone into the feed of sexually undifferentiated fry for a short period from first feeding (Guerrero 1982; Hunter and Donaldson, 1983; Pandian and Varadaraj 1987; Baroiller and Jalabert 1989). In sex-reversal, the hormone administered does not alter the genotype of the fish, but directs the expression of phynotype. Over the years several chemicals (i.e. steroid hormones, hormone analogues and non-steroidal compounds) have been tested and/or used to direct gonadal differentiation of tilapia fry. Administration of the androgen, 17α-methyltestosterone, is regarded as the most effective and economically feasible method for obtaining all-male tilapia populations. Sexual differentiation is known to occur in *Oreochromis* fry at or around 17-19 days after hatching (Yamazaki, 1983; Mair and Little, 1991; El-Sayed, 2006). The best results have been achieved at a dosage of 60mg/kg administration for 21-30 days (Guerrero and Guerrero, 1988; Ridha and Lone, 1990; MacIntosh and Little, 1995; Penman and McAndrew, 2000; Beardmore *et al*., 2001). Steroids are not water soluble and are added to the diet by dissolving an appropriate quantity of hormone in alcohol, or fish or vegetable oil to prepare a stock solution. Androgens such as methyltestosterone (MT) dissolve readily in ethanol and a stock solution using 95 to 100% pure ethanol can be prepared at a strength of 6 g/L. Ten mL of stock solution added to a carrier and mixed with 1 kg of diet would be adequate to prepare a diet to obtain 60 mg MT/kg of diet (Phelps and Popma, 2000).

The success of sex-reversal, measured in terms of the percentage of males, is largely dependent on the intake of hormone treated feed. Hormone intake via the feed is influenced by many factors (Mair and Little,1991; El-Sayed, 2006) such as 1) quality of treated feed, composition of the raw ingredients, method of preparation and storage; 2) palatability and particle size (both affect actual intake of first-feeding fry); 3) feeding frequency and mode e.g. ad libitum or, percentage of body weight and number of times feed is given; 4) water quality and temperature, dissolved oxygen level, pH and algal composition of the water; 5)

stocking density; 6) availability of treated food to all fry; 7) other logistical problems such as reliable refrigeration and storage.

The use of this technique, however, has not been fully accepted in some countries due to environmental and social constraints. Baroiller and Jalabert (1989) reported that the effect of the degradation of synthetic androgen on the metabolism of fish and the environment was not fully understood. Lückstädt (2006) indicated that the United States Food and Drug Agency does not approve the sale of hormonal (steroid) treated fish. El-Sayed, (2006), maintain that the use of hormones for sex reversal is under increasing public criticism, largely, due to perceived potential health and environmental impacts, and social constraints. Fitzsimmons (2007) explained that the hormone, 17α-methyltestosterone, commonly used for sex–reversal is not detectable in the fish and the environment 90 days after cessation of the 21-30 days administration of sex-reversal diet, and even less so towards the harvesting of Tilapia is 4-6 months later. This is also the basis of approval of the use of 17α-methyltestosterone and the method of hormonal sex reversal by USA-FDA.

A main concern, however, is in developing countries with regard to the exposure of (female) workers during the handling and administration of androgenic hormones often without protective gear and safe procedures. In developing countries such as in sub-Sahara Africa there is no effective regulations on the use of the hormone while it also remains difficult to acquire due to cumbersome import regulation. It takes between 6-8 months to secure the importation of a controlled substance such as 17α-methyltestosterone (personal experience, 2002).

Chromosome manipulation

The problems associated with the hormone-induced sex-reversal prompted the search for alternative methods for the production of monosex populations of fish species. Beardmore *et al.* (2001) stated that the best way to obtain all-male populations is through genetic control. Chromosome-set manipulation is one such option that has various applications. The occurrence of YY male fish was first shown by Yamamoto as early as 1958 in medaka, *Oryzias latipes* using the technique of hormonal sex reversal and selective breeding. Subsequently, YY males were also produced in guppy, *Poecillia reticulia* (Yamamoto, 1963), in goldfish and *Carassius auratus* (Yamamoto, 1975). YY males were produced in tilapia through technology of ploidy induction (such as induced gynogenesis, androgenesis and induced polyploidy), by radiation or physical shock (i.e. pressure or thermal), *Oreochromis niloticus* and *O. mossambicus* (Varadaraj and Pandian, 1987; Pandian and Varadaraj, 1990).

The concept of a YY male breeding program to produce a monosex population in commercially important tilapia, *Oreochromis niloticus* was developed through extensive research on the genetics of sex determination in tilapia species by the University College of Wales, Swanzea, UK. Studies revealed that *Oreochromis niloticus* displays genotypic system of a male heterogametic (XY) and female homogametic sex (XX) (Penman *et al*., 1987; Shah, 1988; Mair *et al.*, 1991; Müller-Belecke and Hörstgen- Schwark, 1995; Mair *et al*., 1997). The development of YY male technology involves a series of stages of feminization and

progeny testing, as described by Mair *et al.* (1997) to produce a YY male genotype of *O. niloticus*. It was demonstrated that the YY male genotypes are viable and as fertile as normal XY males, with the ability to sire monosex XY genetically progeny with a phenotypic sex ratio of 93-95% males. This approach made possible the production of genetically male tilapia (GMT) on a commercial scale.

The technique can be considered as environmentally friendly because of low level hormone treatment is limited to the broodstock and no hormones are applied to fish that are consumed (Mair *et al*., 1997). The genetic integrity of species or strain is also not affected and the fish produced for culture maintain a normal genotype. The major drawback is the developmental process, which is complex in nature, costly, time consuming and labour intensive. In spite of this Abucay and Mair (2000) contend that the YY male line has further, been selected for improved growth rate and for GMT sex ratio, through intensive within-family selection in a synthetic female line. According to Mair *et al.* (1997) all-male Nile tilapia, based on the YY-GMT technique is, extensively cultured in the Philippines since 1995 and in Thailand since 1997. Lower levels of uptake have been observed in countries such as China, Fiji, Vietnam, Central American countries, and USA. The major drawbacks associated with the application of genetic manipulation, however, remain highly relevant in Sub-Saharan Africa.

2.6 Influence of environmental substances on Tilapia reproduction

It is, well established that sexual maturity in teleost fish such as tilapia is a function of age, size and environmental conditions. Studies overtime indicate that tilapia populations in natural large lakes mature at a later age and larger size than the same species raised in small farm ponds (Rana, 1988; Popma and Lovshin, 1995; Popma and Masser, 1999; Popma and Shelton, 2006).

Tilapias spawn several times in equatorial and tropical freshwater bodies of Sub-Saharan Africa (SSA), where temperature is high all year round. Siddiqui (1979) reported that tilapia breeding in Lake Navash, Kenya, is non-seasonal and individual fish spawn successfully all year round. Tilapia has a high level of fry survivorship due to apparent parental care. Guarding the young increases the survival of the fingerlings and reproductive success. Studies have shown that, just like other aquatic organisms in the wild, tilapia populations are maintained within the carrying capacity of the water body by environmental pressures such as temperature, food availability and social factors, such as cannibalism from older generation (i.e. fingerlings attacking larval fish), predation from piscivores such as catfish (*Clarias* spp and *Heterobrancus* spp) and perches (e.g. Nile perch). This biological control by piscivores has been exploited in aquacultural set-up in the form of polyculture (Mair and Little, 1991; Popma and Lovshin, 1996). Thus tilapia populations in the wild are maintained by the environmental conditions including predation (both man and other vertebrates), competition (i.e. both intra and inter), natural causes (such as diseases) and other social interactions.

Fish populations are also affected by other environmental residues that come from human activities, producing a huge volume of pollutants. These pollutants such as agricultural by-products, industrial waste

and untreated sewages drain into streams, rivers, lakes and other aquatic water bodies through precipitation and dry deposition, by stormwater transport of fertilizers, and road silt and direct disposal (Allan, 1995). According to Mills and Chichister (2005), since the publication of the book “Our Stolen Future” in 1996, it has become clearer that pollution of the aquatic environment is affecting its wildlife population. The authors maintain that over the years the aquatic environment has been considered as “the ultimate sink” for natural and manmade chemicals, receiving all manner of constituents from the terrestrial ecosystem.

Water bodies are subjected to anthropogenic pressures affecting the reproductive status of fish populations. Species with special demands for environmental conditions, including water quality and spawning sites will have to respond to changes of the ecologicaly (Pavlov *et al*., 2009). Studies on pollution’s effect on parental care in fish, show a decrease in nest building activity and increase in courtship duration, typical of tilapias, and thereby could affect reproduction. Turbidity, which is a product of environmental residue that enters water bodies, has been found to affect fish reproduction as vision is considered one of the primary cues in reproductive success in teleost fishes such as tilapias (Siddiqui, 1979).

Several studies over time have shown that some natural and man-made compounds that enter water bodies are toxic to aquatic life. It is now generally accepted that most of these compounds from the industries, sewage systems and agricultural by-products possess the capacity to interfere with endocrine systems and hormonal activities of all animals particularly the vertebrates. These compounds with the ability to interfere with normal hormonal activity in animals are known as endocrine disrupting compounds (or chemicals, EDCs). In the aquatic environment, a variety of exposure routes exist via which EDCs can easily become bioavailable to fish including; aquatic respiration, osmoregulation, dermal contact with contaminated sediments, the ingestion of contaminated food and maternal transfer of contaminants in lipid reserves of eggs or yolk (Jobling *et al*., 1998; Mills and Chichester, 2005; Vajda *et al*., 2011).

Investigations have established that fish populations that have been constantly exposed to EDCs are experiencing changes in population structure. The flexibility in the sex-determining systems in fish makes some species sensitive to environmental pollutants or any compound capable of mimicking or disrupting sex hormone activity (Devlin and Nagahama, 2002; Pavlov *et al*., 2009). Evidently, endocrine disruption and reproductive impairment will have inpact on ecological sustainability of fish populations. Such endocrine disrupting compounds may impact on larval and other developmental stages of fish, causing disruption of sexual development, fertility and behaviour (Mills and Chichester, 2005; Vajda *et al*., 2005). Studies both from the field, as well as laboratory experimentations, have established that fish with continuous exposure to EDCs experience alterations of their reproductive physiology and morphology which include changes in sex ratios, incidence of reproductive defects in larvae, egg-producing cells in the male testis (testis – ova or ovo - testis), reduced gonadal (i.e. testis and ovary) growth rates and size, reduced female reproductive tracts, feminisation of reproductive ducts, reduced gamete quality, degeneration of testicular germinal cells, increased liver size and increased levels of vitellogenin (egg-york protein) in male blood plasma (Jobling *et al*., 1998; Mills and Chichester, 2005; Nash *et al*., 2006; Vajda *et al*., 2011).

2.6.1 Endocrine disruption substances

According to Evans *et al.* (2011) the World Health Organization (WHO) and International Programme for Chemical Safety (IPCS- which involves WHO, United Nations Environmental Programme, UNEP and International Labour Organization, ILO) define an endocrine disrupter as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations". An endocrine disrupting compound (EDC) is a substance that, when absorbed into the body, has the capability of disrupting the organism's normal functions by affecting hormone production, transport, or response. The authors reported that WHO/IPCS describe a "potential endocrine disrupter as an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations ".

Reports from studies such as Jobling *et al.* (1998), Pait and Nelson (2002), Mills and Chichester (2005) and Lyssimachou (2008) have led scientists to believe that EDCs mode of action is employed through one or a combination of these routes which include:

(i) mimicing the biological activity of a hormone by binding to a cellular receptor, which could lead to an unwarranted response by initiating the cell's normal response to the naturally occurring or endogenous hormone such as the estrogens and androgens at the wrong time or to an excessive extent (agonistic effect).

(ii) binding to the receptor but not activate it, in this case the presence of the chemical on the receptor will prevent binding of the natural hormone (antagonistic effect).

(iii) binding to transport proteins in the blood, thereby, altering the levels of natural hormones that are present in the circulation.

(iv) interfering with the metabolic processes in the body, thus, affecting the synthesis or breakdown rates of the natural hormones, therefore, it alters the pattern of synthesis and metabolism of normal hormones.

(v) interfering with the binding proteins that act to transport endogenous hormones to their destination.

Endocrine disruption occurs through the interaction of exogenous compounds with internal endocrine signalling pathways in an organism through one or a combination of the mechanisms stated above. Such EDCs simulate the effect of endogenous hormones, thereby interfering with normal hormonal activity which alters the natural hormone synthesis and metabolism.

A wide range of compounds (or chemicals) either natural or artificial enter watercourses which can affect the gonads and other parts of the endocrine system. This causes decreased fertility, abnormal sexual differentiation and behavior, decreased response to stress, immune deficiency or altered basal metabolism. Chemicals that have been reported as EDCs include pharmaceuticals and synthetic hormones (such as ethynylestradiol; found in contraceptive pill), dioxin and dioxin-like compounds; industrial chemicals and pesticides (such as, polychlorinated biphenyls, fenoxycarb, polybrominated diphenyl ethers (PBDEs),

dichloro-diphenyl-trichloroethane (DDT)) and plasticizers such as bisphenol A and nonylphenol; and herbicides (including atrazine, diazinon, and permethrin). These chemicals can impact negatively on vertebrates including mammals, birds, reptiles, amphibians and fish (Mills and Chichester, 2005; Takurah and Takemura, 2011).

The classes of compounds (or chemicals) that show characteristics of endocrine-disruption, tend to affect the development and reproduction of organisms by interfering with normal synthesis, secretion, storage, release, transport, metabolism, binding, action or elimination of endogenous or natural hormones in the body, which are responsible for reproduction, development, behaviour and the maintenance of homeostasis (Jobling *et al*., 1998; Mills and Chichester, 2005; Lyssimachou, 2008). Tissues and receptors that participate in hormone biosynthesis, transport, activity, and metabolism in particular are, affected by EDCs. Tabb and Blumberg (2006) reported that EDCs of environmental and dietary origin have the capacity to interact with the estrogen signalling pathways at very low doses. According to Vadja *et al.* (2008) it is, established that when an organism is expose to exogenous endocrine active compound during the critical life stages the organism's development and function can be disrupted.

Endocrine disruption also can be caused by naturally occurring chemicals or products (human and animal waste, e.g. estrogen), which could be steroidal or non-steroidal (Sassi-Messai *et al*., 2009; Vadja *et al*., 2011). Medicinal supplements, such as those recommended for estrogen replacement therapy in post-menopausal women contain high levels of phytoestrogens. Industrial activities such as pulp and paper production also can release large quantities of phytoestrogens which have been found to affect reproductive system and function in fish. The degradation of vegetable matter and paper products in wastewater treatment plants also may contribute to releases of phytoestrogens into the aquatic ecosystem (Turker and Takemura, 2011). One major by-product that comes out of pulp and paper mill is β-Sitosterol (a major plant sterols) which is linked to induced production and increased levels of vitellogenin in fishes (Pait and Nelson, 2002).

2.7 Endocrine disruption

The endocrine system of all vertebrates is important for the control and regulation of all the major functions and processes of the body which include energy control, reproduction, immunity, behaviour (e.g. fight or flight response), growth and development. Normal functioning of the endocrine system encompasses a wide fluctuation of hormonal and other biological indices. For example, extreme concentrations of sex hormones occur at specified times for normal physiological functions; such as sexual differentiation, puberty, and reproductive cycles (Nagahama, 1994; Okuzawa, 2002; Mañanós *et al*., 2009). Endocrine disruption compounds (EDC) on the developmental and reproductive aspect of an organism occurs when the EDC is capable of altering biosyhthesis, release, transporting, binding, storing, affecting metabolism and / or eventual elimination of natural hormones.

The environmental chemicals that find their way into aquatic environment called xenoestrogens or EDCs including ethinylestradiol-17α, nonylphenol, polychlorinated biphenyls and PCB can act as endogenous estrogen and adversely affect the hormone system of teleost fishes. In addition a huge volume of plant derived substances called phytoestrogens are also present naturally either in the environment or as dietary ingredients (Turker and Takemura, 2011). Studies indicate that effectiveness of EDCs to elixit any effect on an individual in the aquatic environment is dependant on a number of factors including exposed life stage of the organism, the season, EDC concentration, duration of exposure, bioconcentration, presence of other EDCs, presence of other environmental stressors present such as temperature and salinity, movement and location (Pait and Nelson, 2002; Mills and Chichester, 2005).

According to Nagahama (1983) estrogens are the steroid hormones that control the important reproduction related processes, including sexual differentiation and maturation. Afonso *et al.* (2001) reported that in fish estrogen biosynthesis is mediated by the steroidogenic enzyme cytochrome P450 aromatase, which converts androgens to estrogens. These authors referred to the main functions of estrogens as the control of sexual differentiation, maturation and reproduction. In addition, these hormones also affect other processes of development, homeostasis and differentiation. Studies have shown that estrogens are also involved in the regulation of the cell cycle and growth, including uterine growth in mammals, neuronal growth and gonadal differentiation in teleost fishes (Nagahama, 1994; Strüssmann and Nakamura, 2002).

Studies have established that the most commonly reported reproductive problems associated with endocrine disruption compounds (or chemicals) in wild fish species include (i) retarded or disrupted testicular development (Lye *et al*., 1997), (ii) testis-ova fish (i.e. egg producing cells in a male testis) (Jobling *et al.,* 1998), (iii) elevated levels of a female egg protein(i.e. phospholipoprotein vitellogenin) in male fish (Janssen *et al*., 1997; Jobling *et al.,* 1998), (iv) degeneration of gonadal tissue (Janssen *et al*., 1997; Lye *et al*., 1997), (v) masculinization of females (Jobling *et al*., 2002), and (vi) anomalous reproductive behaviour (Jobling *et al*., 2002). Other testicular abnormalities documented in some of the fish exposed to EDCs include feminized or absent vas deferens and impaired milt production in male fish (Jobling *et al*., 1998; 2002).

According to Devlin and Nagahama (2002) fish has a plastic sex-determination system, which makes some species sensitive to environmental pollutants or exogenous compounds capable of mimicking or disrupting sex hormone actions. Available information on the effect of EDCs on fish suggests that the threat on reproductive health is, more pronounced in males (Jobling *et al*., 2002). Male fish losing its sterility can lead to failed spawning opportunity for females. Pavlov *et al.* (2009) stated that anthropogenic factors such as EDC and pollutants can affect gametogenesis leading to a disturbance of normal reproduction and appearance of unviable progeny. Accordingly, some of the most usual anomalies in the reproductive cycle of wild fish include asymmetrical development and morphological deformation of the gonads, retardation of sexual maturity, change in the duration of the periods of gonadal development, reabsorption of sex cells in females and males at various stages, increase in the number of fishes omitting spawning, and decreased fecundity. The authors showed that the reproductive system reacted differently to the influence of negative factors, which could be seen by the change in the regeneration properties of sex cells at various stages of

their development. A predominance of cells at mitotic or meiotic divisions, which are the most sensitive, can lead to a total sterilisation of the gonad. Owing to regeneration properties of the reproductive system in fish, the reproductive strategy can change under the influence of anthropogenic pressure, which enables the fish to protect its population from extinction.

The state of fish gonad and reproduction are regarded as the more credible or reliable indictors of endocrine disruption in aquatic systems by both natural and artificial chemicals (Jobling *et al.,* 1998; Celino *et al.,* 2009). Turker and Takemura (2011), intimated that adverse human health effects from EDCs are unlikely to be caused by estrogenic compounds in water, because their concentrations in water appear to be minute in comparison with those due to phytoestrogens and other estrogenic compounds existing in food sources (Mazur *et al*., 1998). In terms of consumption human are less likely to be affected adversely by estrogenic compounds present water bodies when compared with fish which are constantly been exposed to to EDCs present in the aquatic environment.

2.7.1 Phytoestrogen as Endocrine Disruption Substances

A phytoestrogen may be considered as “any compound that induces biological responses in vertebrate, by mimicing and / or modulating the actions of endogenous estrogens (such as 17β-estradiol) usually by binding to estrogen receptors” (Patisaul and Jefferson, 2010). Estrogens are the natural hormones that ensure establishment and maintenance of reproductive systems in vertebrates. According to Patisaul and Jefferson (2010) the US Environmental Protection Agency (EPA) described “phytoestrogens as naturally occurring plant compounds that are structurally and/or functionally similar to mammalian estrogens and their active metabolites”. The authors further explained that these are compounds which have the capacity to alter the structure or function(s) of the endocrine system and cause adverse effects including; the timing of puberty, capacity to produce viable and fertile offspring, sex specific behaviour; premature reproductive senescence, and compromise fertility.

The main classes of phytoestrogens are the flavonoids (quercetin, kaempferol), isoflavones (genistein, daidzein, glycitein, equol and biochanin A), the lignans (enterolactone, enterodiol), the coumestanes (coumestrol), the stilbenes (resveratrol), saponins and the triterpenes (e.g. oleanolic acid and ursolic acid). These compounds are all polyphenols whose molecular structure may or may not be similar to, but functionally similar to the natural androgens and estrogens, particularly 17β-estradiol, and show biological activity in human and other vertebrates (Mazur, 2000; Dixon, 2004; Tuan, 2006; Moutsatsou, 2007; Turker and Takemura, 2011), and polyphenols are known to be present in all plants (Manzur, 2000).

According to Manzur (2000) the flavonoids are the most common polyphenol identified in the plant kingdom, and represents more than 5000 compounds. The flavonoids are subdivided into six major classes viz: anthocyanidins (e.g. cyanidin), flavanols (e.g. catechins, epicatechin, gallocatechin), flavones (e.g. apigenin, luteolin), flavanones (e.g. naringenin, hesperidin), flavanones (e.g. naringenin) and isoflavones (e.g. genistein, daidzein). Studies have established that among the flavonoids, it is the isoflavones (i.e. genistein,

daidzein) which exhibit estrogenic effect and some flavones, flavanones and flavonols (apigenin, kaempferol and naringenin) that activate estrogen-receptor-mediated signalling.

Estrogen (particularly, 17β-estradiol) is the most important steroidal hormone in the control of reproduction-related processes, including sexual differentiation, maturation and onset of puberty (Nagahama, 1994; Okuzawa, 2002; Taranger *et al.,* 2010). It is proposed that sex steroids are the natural inducers of the onset of puberty in male African catfish (Schulz and Goos, 1999), so any compound or substance that can mimic the activity of the sex steroids have capability of suppressing onset of puberty in fish.

Evidence of a phytoestrogen exerting an effect on reproductive endocrine function in the wild was found in effluents from pulp and paper mills, and this observation has been repeated in laboratory studies. Tremblay and Van Der Kraak (1999) exposed sexually immature rainbow trout to β-sitosterol (a phytoestrogen found in the pulp and paper effluent) for three weeks, and resulted in the induction of vitellogenesis and contributed to alteration in the fish reproductive development. Pait and Nelson (2002) reported that studies conducted adjacent to a paper and pulp mill discharges revealed that effects in fishes include depressed levels of reproductive hormones, decreased gonadosomatic index (GSI) in both male and female fish and external masculinization of females. Manning (2005) reported that a series of *in vitro* and *in vivo* studies have confirmed that β-sitosterol (a major plant sterol found in pulp mill effluents) can interact with the oestrogen receptor and induce the production of vitellogenin in male fish.

A high plant content diet of some fish, for instance omnivorous tilapias in the wild, and also the use of soybean in the diet of fish in captivity, could be a source of considerable amount of phytoestrogens (Pelissero and Sumpter, 1992). An earlier laboratory study that showed that phytoestrogens can induce production of vitellogen in male and juvenile sturgeon (*Acipenser baeri*) was demonstrated by Pelissero *et al.* (1991a; 1991b).

The exact mechanism by which phytoestrogen elicit an effect is not very clear. However, Cheshenko *et al.* (2008) explained that the general assumption is that the phytoestrogens act by inhibiting steroidogenic enzymes through competitive inhibition with natural substrates for a particular enzyme. Also some phytoestrogens are able not only to bind to respective receptors, but also to directly interact with aromatase CYP19, possibly leading to inhibition of this enzyme involved in the synthesis of 17β-estradiol, thereby affecting reproductive health of the fish. Laboratory studies have shown that although both male and female vertebrates produce and use estrogens and androgens, i.e. estrogen plays a role in reproductive activity in males such as the regulation of GTH secretion by the pituitary gland (Pait and Nelson, 2002). Thus, any substance that can interfere with the activity of either estrogens or androgens, can lead to defect in the reproductive health of the fish.

There is evidence that exposure of an organism to natural hormones or EDCs that may affect the functioning of the endocrine system, could also influence the reproductive endocrine function and cause changes in

reproductive development (Jobling *et al.*, 1998; Damstra *et al.*, 2002; Manning, 2005; Mills and Chichester, 2005; Cheshenko *et al.*, 2008; Sassi-Messai *et al.*, 2009; Blazer *et al.*, 2012).

2.7.2 Medicinal plants as endocrine disrupters

The most commonly reported phytoestrogens identified in the major known edible and medicinal plants include flavonoids (flavonols, isoflavonoids, and isoflavans); coumestans (coumestrol); lignans; stibenes, β-sitosterols saponins and the triterpenes.

The effect of phytoestrogens on fish was initially documented / studied in trout and sturgeon (Pelissero and Sumpter, 1991; Pelissero *et.al,* 1991; Pelissero *et al.,* 1996). Pelissero *et al.* (1991) assessed the estrogenic activity in daidzein, genistein, equol and coumestrol by their induction of hepatic synthesis of vitellogenin secretion in yearling Siberian Sturgeon. The result indicated that coumestrol appeared to be the most potent compound, inducing the most vitellogenin secretion with the lowest dose administered. Kiparissis *et al.* (2003) exposed Japanese Medeka (*Oryzias latipes*) to equol (a product of intestinal digestion of diadzein) soon after hatching. The treatment induced gonadal intersex (i.e. testis-ova) in males within approximately 100 days.

Attempts, both in the laboratory and in the field have been conducted for the control of precocious breeding in tilapias. The saponins which are steroidal glycoside linked to hydrophobic aglycone (sapogenin) can be an alternative to androgenic hormones used for tilapia sex inversion and sterility. Preliminary laboratory and field trials of Quillaja saponin-supplemented diet (Saponin - a glycoside linked to hydrophobic aglycone – sapogenin), on *Oreochromis niloticus* indicates potential of saponin suppressing tilapia reproduction (Francis *et al.*, 2005). Studies on the effect of saponin on the reproductive activity of tilapia showed possible infertility in females when fed with a diet containing 300 mg/kg saponin; sex inversion to all male populations at 700 mg/kg saponin inclusion; and a higher number of males noted in those fish fed with 150-500 mg/kg saponin diet when reared under laboratory conditions. These positive results of saponin in aquaria experiments however required testing under pond production conditions to ascertain its benefit to commercial aquaculture (Lückstädt *et al.*, 2006; Kühlmann *et al.*, 2006).

2.8 Alternative Approaches to Control of Reproduction in Farmed Tilapia

The most widely used strategies to produce monosex populations include hand-sexing/sorting, hybridization, hormone-induced sex reversal and chromosome manipulation, each with its own shortcomings or limitations. Hormone mediated sex reversal is the most widely practised monosex production technology. Concerns with regard to human health, food safety and environmental impact are fuelling the need for alternative methods that offer non-hazardous, consumer and environment-friendly ways for the production of monosex populations of aquaculture species.

2.8.1 Thermally Mediated Sex Reversal

Studies of the underlying mechanisms of sex determination in animals, including fish species, confirmed the influence of environmental factors on sex ratio, particularly during the stage of sexual differentiation. The effect of temperature on sex differentiation was first observed in reptiles. Temperature directed sex differentiation has been observed in turtles (Bull, 1980; Bull and Vogt, 1981) where the sex differentiation is influenced by incubation temperature, with low temperatures resulting in males and high temperatures resulting in females (Bull, 1980).

Water temperature has been shown to influence the sex ratio of fish (Mair *et al.*, 1990; Baroiller *et al.*, 1995; Depsrez and Melard, 1998; Abucay *et al.*, 1999; D'cotta *et al.*, 2001) including tilapia (Mair *et al.*, 1990; Baroiller *et al.*, 1995; Mbahinzireki and Dabrowski, 1997; Abucay *et al.*, 1999; Baroiller and D'Cotta, 2001). In Atlantic silverside (*Menidia menidia*) sex determination has been found to be temperature dependent (Conover, 1984). In tilapia, Mair *et al.* (1990) reported that at a cold temperature of 19^{o}C the sex ratio of *Oreochromis mossambicus* displayed excess females (89%) in comparison to the control reared at 28^{o}C (50%). Mbahinzireki *et al.* (1997) also demonstrated a shift in the sex ratio of *O. niloticus* progeny reared at 35 ± 1^{o}C for a period of 60 days as 80% male compared to 1:1 sex ratio of the control reared at 22 ± 1^{o}C. Abucay *et al.* (1999) also reported on the effect of temperature on sex differentiation in *O. niloticus*. These authors maintain that high temperature can affect both males and females and therefore concluded that susceptibility to temperature effect on sex ratio appears to have genetic basis.

Although a possibility exists of using thermal treatment to influence gonadal differentiation in tilapias and thereby manipulate sex ratios, these methods to date lack the sufficient control and complete reversal that is required to ensure commercial application.

2.8.2 Use of Medicinal Plants

Natural reproduction inhibitors found in medicinal plants provide an alternative for the control of sex ratio in fish. Farnsworth *et al.* (1975) described medicinal plant as "any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi synthesis". Such a plant will have its parts including grains or seeds, flowers, fruits, stems, barks, rhizomes and roots employed in the control or treatment of a disease condition on the basis that it contains chemical components that are medically active (Farnsworth *et al.*, 1975; Soejarto, 1978). These non-nutrient plant chemical compounds or bioactive components are referred to as phytochemicals or phytoconstituents and are responsible for protecting the plant against microbial infections or infestations by pests (Abo *et al.*, 1991; Liu, 2004; Nweze *et al.*, 2004; Doughari *et al.*, 2009).

When evaluating various plants or their extracts for fertility regulating agents, after oral in vivo treatment (in animal models, e.g. rat), utero-tonic effects could show anti-implantation, estrous cyclic disruption, anti-luteinizing hormone (LH) activity, luteolytic activity and luteal suppressant activity (Soejato *et al.*,1978). Reports indicated that medicinal plants have been used to induce sterility in laboratory animals (Gary and

Garg 1971; Bodharkar *et al.*, 1974; Das, 1980) with much success. This finding stimulated research to assess the alternative use of natural reproductive inhibitory agents in some plants to control tilapia fertility in ponds.

Various drugs of plant origin have been identified that influence the human reproductive system through an anti-ovulatory effect, union of ova and sperm, as an abortifacient (i.e. induces the premature expulsion or abortion of the foetus), as well as have an anti-implantation effect (Unny *et al.*, 2003; Kamal *et al.*, 2003). The effectiveness of plant based male contraceptives have been tested on various laboratory animal models such as rat, albino rats, rabbits, mouse, dog, guinea pigs, hamster, monkey (langur & bonnet) and on human sperm. Sharma *et al.* (2001) mentioned that production of male contraceptives have been directed towards the development of anti-spermatogenic agents to suppress sperm production and maturation, transport through the vas deferens and deposition. Up to 200 plants have been evaluated for their antifertility properties, with the type of plants, their extracts, dosage, animal model and pharmacological activity described by Kamal *et al.* (2003) and Unny *et al.* (2003).

2.8.3 Use of Phytochemicals with Antifertility/Abortifacient Activity

Fahey (2005) described phytochemicals as “chemicals produced by plants which may have an impact on health, but which are not required by humans as essential nutrients”. Kumar *et al.* (2012) listed 577 plant species with bioactive chemicals, which show promise as alternative oral fertility regulating agents in males and females (i.e. humans). These authors maintained that isolated phytoconstituents like abridine, aristocholic acid, butin, embelin, ferujol, oleanolic acid-3-glucoside, p-Coumaric acid, and vicolide B have shown 100% antifertility activities, whereas acacetin, luteolin, momorcharins, piperine, plumbagin, sitosterol, yuanhuatine, yuehchukene also significantly inhibited fertility. Despite the extensive studies, the authors maintained that the mechanisms of action by which plant extracts and their active compounds exert antifertility effects remain to be studied.

Studies have shown that some phytochemicals are natural steroid-like substances with estrogen-like biological activity, and are thus called phytoestrogens (i.e. estrogenic compounds found in plants).

2.9 Classes of Phytoestrogens

Ososki and Kennelly (2003) defined phytoestrogen as “any substance or metabolite that induces biological responses in vertebrates and mimic or modulate the actions of endogenous oestrogens (such as 17β-estradiol – Figure 2.6), usually by binding to estrogen receptors”. Phytoestrogens are believed to exert estrogenic effects on the central nervous system, induce estrus, and stimulate growth of the genital tract of female animals (Murkies *et al.*, 1998, Ososki and Kennelly, 2003). Most of these phytoestrogens have a molecular structure which is similar to the natural oestrogen 17β-estradiol (Figure 2.5).

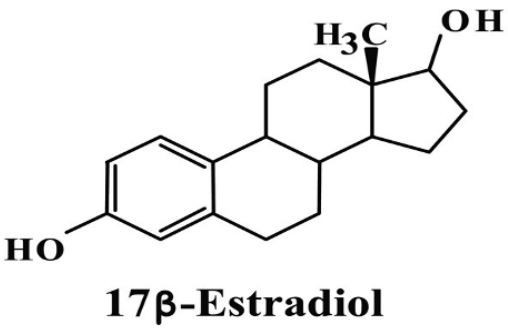


Figure 2.5 The chemical structure of 17β-Estradiol

A summary of dietary phyto-estrogens is presented in Figure 2.6.

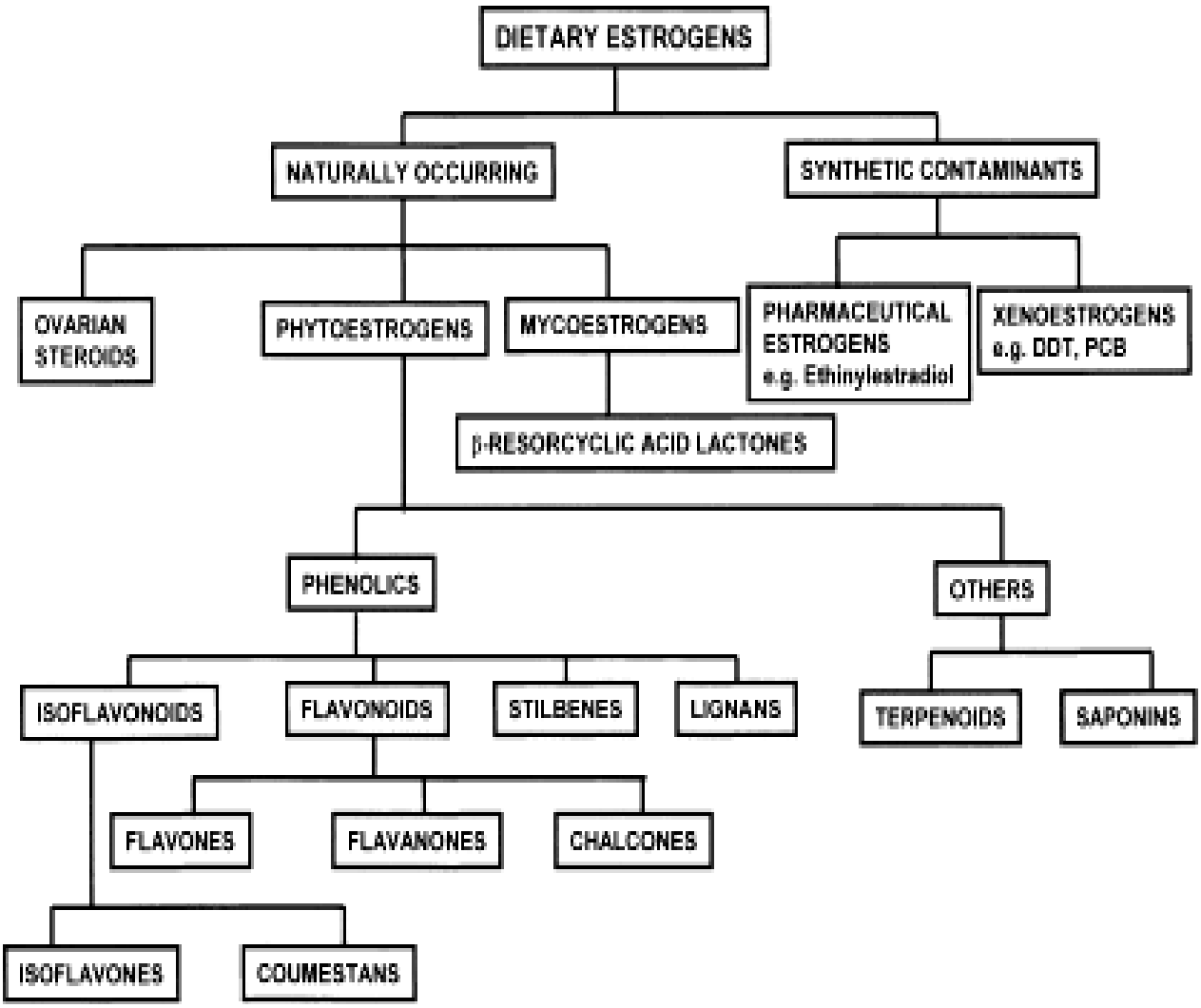


Figure 2.6 Classification of phyto-estrogens/dietery estrogens (adpated from Cos *et al.*, 2003).

Price *et al.* (1985) maintain that the biological interest in phytoestrogens has soared because they exhibit both weak *in vitro* and *in vivo* estrogenic and anti-estrogenic actions. However, like steroidal oestrogen, these plant estrogens have the potential to exert adverse as well as beneficial effects and, in particular, the dietary intake of phytoestrogen has been reported to influence reproductive physiology in animals. Recognition of the endocrine disrupting properties of phytoestrogens dates back to the 1940's when ewes grazing on a rich clover (i.e. legume of family- Fabacae, UC-ARN 2007) pastures in Australia were observed to have high rates of aborted foetus, reproductive abnormalities in their offsprings and abnormally high rates of infertility. Also, it was discovered that prolonged grazing on clover plants resulted in permanent infertility in sheep (ewes) and cows (Bennets *et al.*, 1946; Adams, 1981, 1990, 1995; Price *et al.*, 1985; Adams and Sanders, 1988). In searching for the cause of the observations associated with the eating of clover, scientists identified the coumestrols, isoflavones, genistein and daidzein and their precursors biochanin A and formononetin as components of the clover (Adams 1981; Price *et al.*, 1985; Kurzer and Xu, 1995; 1997). Setchell *et al.* (1987) reported liver disease and infertility in captive cheetahs placed on a soy-based diet, which was, concluded to emanate from isoflavones. Since this discovery several reports have emerged that over 300 plants possess properties that cause estrogenic activty in animals, and several studies have been undertaking to identify phytoestrogens present in animal and human food products (Farnsworth *et al.*, 1975; Price *et al.*, 1985; Kurzer and Xu, 1997).

2.9.1 Commonly Known Phytoestrogens

Phytoestrogens generally, include a number of classes of compounds, which include the flavonoids (flavonols, isoflavans and isoflavonoids), coumestans (coumestrol), and lignans. Several of these compounds have, been identified in vegetables, fruits and whole grains commonly consumed by humans. According to Franke *et al.* (1994) the growing interest in phytoestrogens stemmed from findings in many cell-line and animal models which suggested that these phytochemicals can act as cancer-protecting agents. Other properties connected with cancer prevention such as antioxidant, radical scavenging, serum cholesterol lowering, hypolipidemic, anti-estrogenic and antiproliferative were also observed. Phytoestrogens are present in a variety of plants and are most abundant in leguminous plants. Legumes are a vital component in almost every diet throughout the world, and many other parts of the plant are edible in addition to the seeds (Kurzer and Xu, 1997; Mazur *et al.*, 1998). From among the diversity of phytoestrogens the flavonoids and coumestans are considered as the most common estrogenic compounds in these plants (Price and Fenwick 1985; Mazur, 2000).

Yao *et al.* (2004) defined flavonoids as a "chemical or substance composed of a common phenylchromanone structure in the C6-C3-C6 skeleton with one or more hydroxyl substituents, including derivatives". Flavonoids are secondary metabolites, present in all terrestrial vascular plants. They are a group of polyphenolic compounds with two aromatic rings in their structure that are joined together by a 3C-oxygenated heterocycle. They are diverse in chemical structure and characteristics, and 400 different varients have been identified. These compounds occur naturally in seeds, flowers, fruits, vegetables, nuts, and bark, and form an integral part of the human diet (Cook and Samman, 1996). The major classes include

flavonols, isoflavones and isoflavans. The flavonoids are reported to exhibit multiple biological effects including; anti-oxidant, antiviral, antibacterial, anti-inflammatory, vasodilatory, anticancer, and anti-ischemic (Procházková *et al*., 2011). In addition, flavonoids are known to have the ability to modulate cytochrome P-450 activity. Flavonoids exert these effects as antioxidants, free radical scavengers and chelators of divalent cations. The various activities of flavonoids are influenced by the number of structural features such as (1) presence of hydroxyl group-OH; (2) double bond between carbons two and three (C2-C3); (3) the carbonyl group at C-4 of the C ring; (4) the methoxyl group and (5) the presence of the sugar moity.

Isoflavones/isoflavanoids have been shown to stimulate uterine growth in the laboratory animals thus exhibiting estrogenic action (Drane *et al*., 1980; Price and Fenwick, 1985). Isoflavonoid consumption has also been associated with permanent infertility in cattle and the biochemical and physiological processes associated with sexual maturation in female rats (Whitten and Naftolin, 1992). Assinder *et al.* (2007) reported that male adult rats exposed to diet of high phytoestrogen content disrupts spermatogenesis and increases germ cell apoptosis reducing testicular sperm numbers. The authors contended that the disruption of spermatogenesis was independent of the hypothalamo–pituitary–testicular axis and is likely to be due to disruption of paracrine and/or autocrine actions of estrogen in the testis.

The effect of phytoestrogens on fish was initially studied in and documented for trout and sturgeon (Pelissero and Sumpter, 1991; Pelissero *et.al*, 1991; Pelissero *et al*., 1996). Since then, several attempts have been made to administer phytochemicals to fish, either in crude or pure form to elicit some effect on reproduction. In the tilapias, e.g. *Oreochromis niloticus* laboratory and field trials have been conducted for the control of precocious breeding in tilapias using saponin (Francis *et al*., 2002; Lückstädt *et al*., 2006).

Shanhan *et al.* (2009) reported that phytoestrogens show various effects on the reproduction of both male and female fish in water bodies where they abound. The authors suggested that they may affect the activities of germ cells, the level of sex hormone, and disrupt sex differentiation. As estrogen-like compounds, they may induce VTG production in both female and male fish. The exact mechanism by which phytoestrogens affect the physiological activity of fish is unknown, but it likely occurs through binding with the estrogen receptors (ERs), changing the metabolism of endogenous steroid hormone and modulating the function of enzymes. Further research is necessary to determine the mechanism through which phytoestrogens affect the fish.

Flavonols: Kaempferol and Quercetin

Flavonols (Figure 2.7) have a central ring of 3-hydroxypyran-4-one, but lack a carbonyl group at position 4. The most abundant flavonols are quercetin and kaempferol (Vicente, *et al*., 2009), but quercetin appears to be the ubiquitously one present in most plant-derived foods and medicines. Of the flavonoids, quercetin is among the substances that have been studied in some detail, and it is found in many food plants including citrus fruits, berries, leafy vegetables, roots, herbs and spices, legumes, cereal grains, tea, and cocoa (Dunnick and Hailey, 1992). Devaraj *et al.* (2011) reported that quercetin, kaempferol and rutin have been

extracted from *Moringa oleifera* Lam. leaves through the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS).

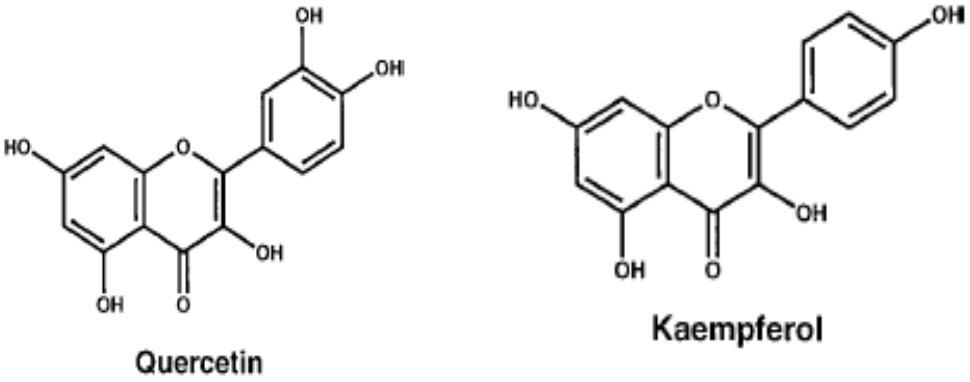


Figure 2.7 The chemical structure of flavonols-quercetin and kaempferol.

Quercetin is commonly found as O-glycosides in which at least one hydroxyl group is substituted by various types of sugars (Figure 2.7); the sugar group is frequently bound at the 4-position. Sousa and Marletta (1985) reported that quercetin is a potent inhibitor of at least three cytochrome P-450-catalyzed reactions in rat liver microsomes *in vitro* (ethoxyresorufin O-deethylation, p-nitroanisole 0-demethylation, and benzo[*a*]pyrene - B(a)P hydroxylation). It interferes with substrate binding and exerts a strong uncoupling effect. The physical properties of quercetin and other flavonols are attributable to their hydrophobic, co-planar structure (Figure 2.7). Their water-solubility increases with increasing number of sugar groups. The chemical activities of quercetin can be ascribed to its electron-donating property (reducing activity) which is due to the presence of a phenolic hydroxyl group (Formica and Regelsos, 1995; Birt *et al.*, 2001).

Møskaug *et al.* (2004) reported that quercetin, has antioxidant activities, inhibits protein kinases, inhibits DNA topoisomerases and regulates gene expression. Quercetin has been shown to protect low-density lipoproteins from oxidation, prevent artherosclerotic plaque formation, and promote relaxation of cardiovascular smooth muscle. In addition, it can inhibit the action of pregnant mare serum gonadotropin on ovarian and uterine growth in juvenile rats, resulting in an arrest in sexual maturation. Quercetin also has antifertility activity by inhibiting sperm motility and the activity of Ca^{2+} - (adenosine triphosphatase – ATPase). It has, also been established that quercetin induces abnormalities in spermatozoa, following injection of quercetin 80mg/kg body weight/day for 5 days or more (Formica and Regelsos, 1995). Quercetin is an attractive natural compound for cancer prevention due to its beneficial anti-mutagenic and anti-proliferative effects, its strong antioxidative capacity, and its role in the regulation of cell signalling, cell cycle and apoptosis were all demonstrated in animal and *in vitro* studies (Murakami *et al.*, 2008).

Kaempferol (Figure 2.7) is a flavonol that have been found in various natural plants including apples, citrus fruits, onions, grapes and red wines. It has also been isolated from tea, mushroom and broccoli (Hertog, 1992). Kaempferol is reported to have several health promoting effects. For example, kaempferol possesses anti-oxidative and anti-inflammatory properties (Burda and Oleszek, 2001; García-Lafuente *et al.*, 2009), and

exhibits anti-tumor activity (Leung *et al.*, 2007). Keampferol possess strong antioxidant properties thereby preventing oxidative damage of cells, lipids and DNA. Studies have confirmed that kaempferol inhibits the formation of cancer cells by acting as a chemopreventive agent (Colino *et al.*, 1999). Rho *et al.* (2011) reported that kaempferol and its rhamnosides derivatives isolated from kenaf (*Hibiscus cannabinus*) possess depigmentation and anti-inflammatory activity.

Isoflavones: Daidzein and Genistein

Isoflavones possess a 3-phenylchroman skeleton derived biogenetically from the 2-phenylchroman skeleton of the flavonoids. Isoflavones are found in Leguminosae plants including soybeans. Isoflavones exhibit some estrogenic and antiestrogenic actions as well as anticarcinogenic activity, both *in vitro* and *in vivo*. It has been reported that it inhibit aromatase enzyme, stimulate sex hormone binding globulin (SHBG) synthesis, manifest in angiogenesis, cell cycle progression, and antioxidant properties (Murkies *et al.*, 1998; Ososki and Kennelly, 2003). The major isoflavones are genistein and daidzein (Figure 2. 8) which occur most commonly in plants as inactive glucosides. Isoflavones may be derived from their precursors, for example genistein from biochanin A and daidzein from formononetin, which are obtained from metabolism after breakdown by intestinal glucosidases. These estrogenically active isoflovones occur exclusively in legumes and beans, particularly, the soybean, and clovers.

The most extensively studied of these two isoflavones *in vitro* is genistein, displaying both estrogenic and antiestrogenic effects on the proliferation of human cancer cell lines. Genistein also exhibits estrogenic action in laboratory animals through stimulation of uterine hypertrophy (Davis *et al.*, 1998). Isoflavone has a molecular and cellular properties similar to synthetic endocrine disruptor, Bisphenol A (BPA), it has therefore provided a useful model to investigate the biological impact of endocrine disruption activity of phytoestrogens. Isoflavones may also have suppressive effects on endogenous estrogen production as a result of aromatase enzyme inhibition and inhibition of 5-α reductase. In rodents, neonatal exposure to genistein alters ovarian differentiation, reduces fertility and causes uterine cancer later in life. Female fertility is also disrupted in rodents following developmental exposure to genistein (Ososki and Kennelly, 2003).

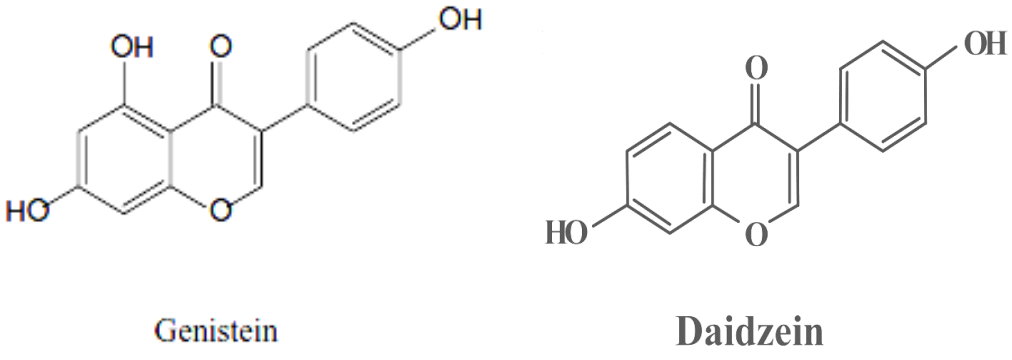
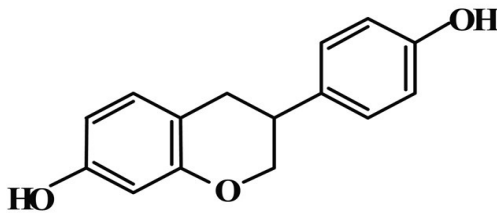


Figure 2.8 The chemical structure of isoflavones: daidzein and genistein.

Isoflavones, genistein and daidzein isolated from soybean have been screened for anti-breast cancer activity on the basis of their influence on estrogen receptors. In a study to assess isoflavones effect on reproduction, genistein and daidzein were injected (0.8 mg daily for 180 days) against N-methyl-N-nitrosourea-induced mammary tumours in Sprague–Dawley rats. The results indicated that genistein and daidzein reduced the number of tumours moderately and marginally reduced tumour incidence (Ososki and Kennelly, 2003). In a study on long-term *in utero* exposure of male rats to genistein during lactation, no apparent adverse effects on gametogenic function and sperm count were found. In human males, a two-month exposure to high dietary genistein levels had no negative impact on sperm function (Roberts *et al.,* 2000; Mitchell *et al*., 2001; Dixon, 2004).

Isoflavans: Equol

According to Carusi (2000) equol (Figure 2.9) is a by-product produced from metabolism of formonetin and daidzein in the gut microflora of farm animal when it consumes a dietary isoflavone from soyabean source. Pelissero and Sumpter (1992) indicated that farmed fish can be exposed to equol which is a metabolite from daidzein through dietary sources because the vegetable components of fish diet often have high levels (> 30%) of soya and alfalfa. Birt *et al.* (2001) reported that laboratory studies with mammalian models have shown that equol and genistein are estrogen agonists.



Equol

Figure 2.9 The chemical structure of the isoflavan, equol

There have been very few studies on the effects of genistein and equol in teleosts. Initial results by Pelissero *et al.* (1991) demonstrated that equol and genistein can interfere with endocrine and reproductive processes in teleosts. By injecting yearling Siberian sturgeon (*Acipenser baeri*) with equol and genistein intraperitoneally (ip), vitellogenesis was induced.

Effects of neonatal exposure to coumestrol and equol on the development of the rat reproductive tract have been examined. When Sprague-Dawley pups were injected subcutaneously with 100µg of coumestrol on postnatal days 1–5, premature uterine gland development and increased uterine weight were observed (Kurzer and Xu, 1997). Kiparissis *et al.* (2003) examined the effect of equol and genistein on gonadal development in the Japanese medaka (*Oryzias latipes*). The fish was exposed to equol 0.4µg/L, soon after hatch. Approximately 100 days posthatch gonadal intersex was induced (i.e., testis-ova) in males at an incidence rate of 10%. Those fish that were treated with equol, 0.8µg/L had an incidence of 87%. However, exposure to 1,000µg/L, genistein caused a low incidence (i.e., 12%) of gonadal intersex in male medaka.

The ovaries of female medaka treated with both equol and genistein showed atretic oocytes, an enlarged ovarian lumen, delayed oocyte maturation, proliferation of somatic stromal tissue, and primordial germ cells.

Coumestans: Coumestrol

The main sources of coumestrol (Figure 2.10) are alfalfa and clover, however, alfalfa sprouts in partucular are considered as the most significant dietary sources of coumestans. Whitten and Naftolin, (1992), showed that feeding a developing female rat with a dietary coumestrol (0.01%), increased oestrogenic activity and suppress estrous cycles. It also has negative effects on the sexual behavior of male offspring, and mutagenic effects were detected. Studies have shown that coumestrol has higher binding affinity for ER than genistein, and it exhibits strong estrogenic activity, similar to that of estradiol.

Besides estrogenic activity coumestrol also effects the metabolism by increasing lipid synthesis and glycogen catabolism in perfused rat liver (Dixon, 2004). Wang et al. (2007), considered coumestrol as a member of the isoflavonoid family, describing it as a plant-derived compound with estrogen-like structure, acting as an antagonist to some classes of cytochrome P450.

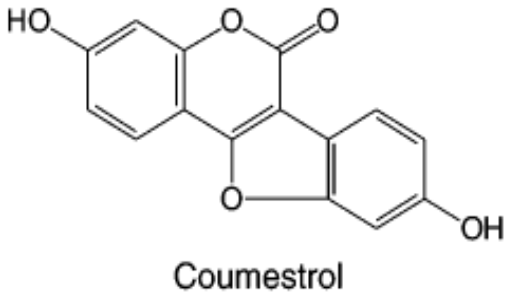


Figure 2.10 The chemical structure of the coumestan, coumestrol

Pelissero *et al.* (1991) assessed the estrogenic activity in daidzein, genistein, equol and coumestrol by their induction of hepatic synthesis of vitellogenin in yearling Siberian Sturgeon. The results indicated that coumestrol appeared to be the most potent compound, inducing the most vitellogenin secretion with the lowest dose administered.

2.9.2 Saponins as Phytoestrogens

Saponins are glycosylated secondary metabolites, non-volatile, surface active compounds, which are widely distributed in nature, and generally occur primarily in the plant kingdom. They are a diverse and chemically structured complex class of compounds that can be divided into three main major groups depending on the structure of the aglycone (Figure 2. 11), which may be steroidal, a steroidal alkaloid or a triterpenoid.

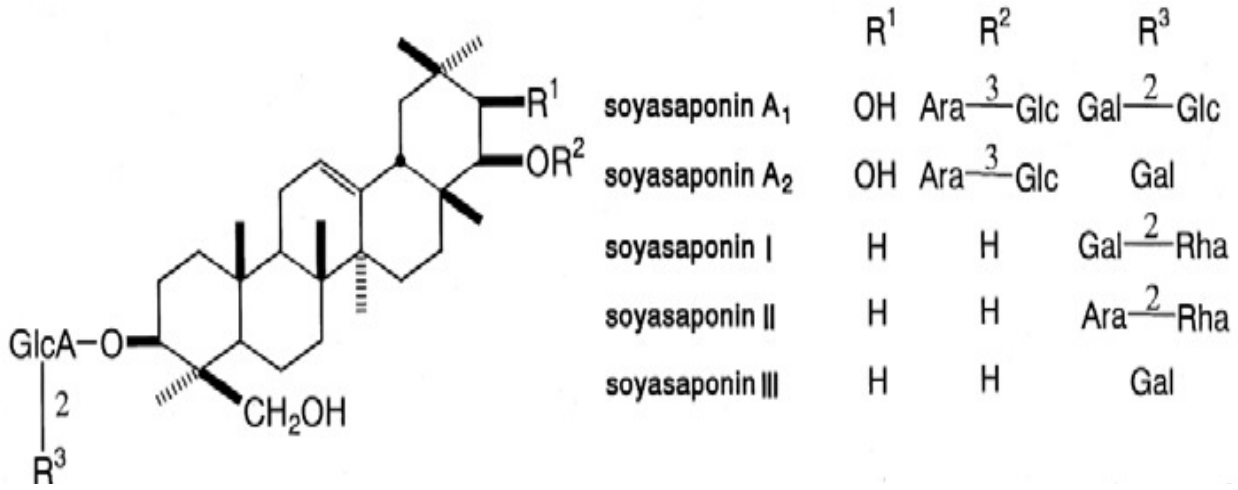


Figure 2.11 The chemical structure of Saponins as isolated from soybean, showing different side chains attached to a triterpenoid backbone (adapted from NSCFS, 2009)

Structurally, saponins are characterized by a skeleton derived from a 30 - carbon precursor oxidosqualene to which glycosyl residues are attached (NSCFS, 2009). Saponin molecules have been proposed to contribute to plant defense, and are also exploited as drugs and medicines and for a variety of other purposes (Osbourne and Haralampis, 2002; Vincken *et al.*, 2007). They consist of non-polar aglycones coupled with one or more monosaccharide moieties. This combination of polar and non-polar structural elements in their molecules explains their soap-like behaviour in aqueous solutions. Saponins can be chemically categorized as comprising of an aglycone linked to one or more sugar chains.

Triterpenoid saponins are predominantly found in dicotyledonous angiosperms and the steroidal glycosides, are almost exclusively from monocotyledonous angiosperms (Price *et al.*, 1987; Sparg *et al.*, 2004; Güçlü-Ustündağ and Mazza, 2007). Their structural diversity is reflected in their biological and physicochemical properties, which are exploited in a number of traditional uses such as soaps, fish poison, and molluscicides and industrial applications (Price *et al.*, 1987; Fenwick *et al.*, 1991; Güçlü-Üstündağ and Mazza, 2007).

Saponins have a diverse range of properties including; sweetness and bitterness, foaming and emulsifying properties, pharmacological and medicinal properties, haemolytic properties, as well as antimicrobial, insecticidal, and molluscicidal activities. It has a wide range of applications in beverages and confectionery, as well as in cosmetics, and pharmaceutical products (Oleszek, 1990; 2002; Oleszek and Stochmal, 2002; Sparg *et al.*, 2004). Dietary saponin is also known to reduce fertility (Qun and Xu, 1998). Gupta *et al.* (2005) reported that saponins of *Albizia lebbeck* bark show antifertility activity in male rats; the activity which may be due to the disturbances induced by the saponins in testicular somatic cell functions.

The saponins which are steroidal glycoside linked to hydrophobic aglycone (sapogenin) can be an alternative to androgenic hormones used for tilapia sex inversion and sterility. Preliminary laboratory and

field trials of Quillaja saponin-supplemented diet (Saponin - a glycoside linked to hydrophobic aglycone – sapogenin) on *O. niloticus* indicates potential of saponin suppressing tilapia reproduction (Francis *et al*., 2005). Studies on the effect of saponin on the reproductive activity of tilapia showed possible infertility in females when fed with a diet containing 300 mg/kg saponin. Sex inversion to all male populations was observed at 700 mg/kg saponin inclusion in feed, and a higher number of males noted in those fish fed with 150-500 mg/kg saponin diet when reared under laboratory conditions. These positive results of saponin in aquaria experiments require testing under pond production conditions to ascertain its benefit to commercial aquaculture (Lückstädt *et al*., 2006; Kühlmann *et al*., 2006).

Most saponins that readily dissolve in water are poisonous to fish, and are believed to be active components of many traditionally used fish poisons, like mahua oil cake (Francis *et al.,* 2001; 2002). According to Roy *et al.* (1990), when saponin is added to water it becomes highly toxic to fish because of the damage caused to the respiratory epithelium of the gills by the detergent action of the saponins. De *et al.* (1987) reported that tea (*Camellia sinensis*) seed cake, containing about 7–8% saponins, which, when added to water at a dose of 100 ppm resulted in the death of tilapia within 5 to 6 hours. Francis *et al.* (2001) also maintained that because of the high solubility of most saponins in water, aqueous extraction would remove most saponins from feed ingredients, and this could be recommended for removing saponins when using saponin containing material for livestock feed.

2.9.3 Triterpenes: Oleanolic acid and Ursolic acid

Oleanolic acid (3β-hydroxy-olea-12-en-28-oic acid) and its isomer ursolic acid (3β-hydroxy-urs- 12-en-28oic acid) are ubiquitous triterpenoids (Figure 2.12) found in the plant kingdom, and are an integral part of the human diet (Liu, 2005; Pollier and Gooseens, 2012). These triterpenoid compounds which are widely found in plants, occur in the form of free acid or aglycones for triterpenoid saponins (Price *et al*., 1987; Mahato *et al*., 1988). Oleanolic acid (OA) as a pentacyclic triterpene is present in many fruits and vegetables, such as olive leaves (*Olea europaea*), mistletoe sprouts (*Viscum album*), grape (*Vitis vinifera*), clove (*Syzyium aromaticum*) and pomegranate (*Punica granatum)* flowers. Ursolic acid has been found to be abundant in *Silphium* species for instance, *S. integrifolium* and *S. trifoliatum* (Kowalski, 2007).

High pressure liquid chromatography with electron impact mass spectrometer detection ((HPLC–EI/MS) or ultra high pressure liquid chromatography with electron impact mass spectrometer detection (UHPLC-EI/MS-MS) has been found to be the most powerful method for the separation, quantification and structural determination of position isomers with very similar structures, such as, oleanolic acid and ursolic acid (Figure 2.12) (Wang *et al*., 2008; Liang *et al*., 2009; Scheinder *et al*., 2009; Csuk and Siewert, 2011, Pollier and Goossens, 2012).

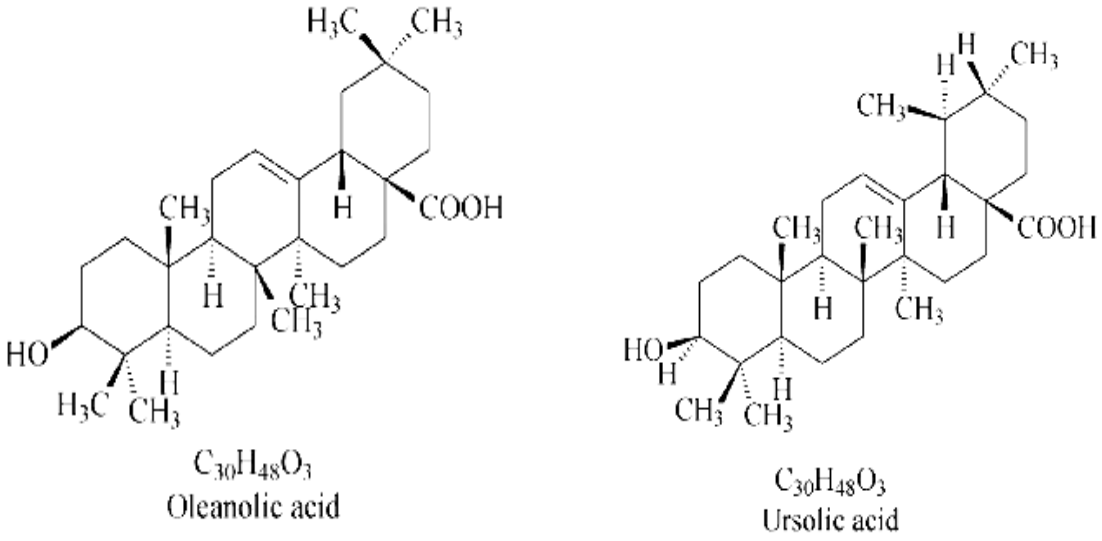


Figure 2.12 The molecular structure of the triterpenoids, oleanolic acid and ursolic acid (adapted from Wang *et al.*, 2008)

Pharmacological studies of oleanolic acid and ursolic acid indicate that these two triterpenoids have many beneficial effects including antibacterial, antifungal, insecticidal, anti-HIV, complement inhibitory, diuretic, antidiabetogenic, and gastrointestinal transit modulating activities. They also have protective action to liver such as anti-inflammatory effects, antitumor activity, and immunomodulatory. These acids have also been shown to act at various stages of tumor development to inhibit tumor initiation and growth, as well as to induce tumor cell differentiation and apoptosis (Liu, 1995; 2005; Kawalski, 2007; Pollier and Gooseens, 2012).

The antifertility activity of oleanolic acid ($C_{30}H_{48}O_3$) isolated from the flowers of *Eugenia jambolana,* was evaluated in male albino rats. The administration of the compound for 60 days reduced fertility of the animals without an effect on body weight or reproductive organs. The compound caused arrest of spermatogenesis but did not induce any abnormality to spermatogenic cells, Sertoli cells or Leydig interstitial cells. The study concluded that oleanolic acid appears to be a promising antifertility agent devoid of undesirable side effects (Rajasekaran *et al.*, 1988).

Oleanolic acid and other triterpenoids have been shown to be an inhibitor of testosterone 5α-reductase (Ohyo, 1985). A 30-day administration of oleanolic acid was adequate to produce reversible sterility in male Wistar rats, without adverse effects on *libido (*Mdhluli and Van der Horst, 2002). Das *et al.* (2011) evaluated the spermicidal activity of oleanolic acid 3-β-D-glucuronide (OAG), an active principle isolated from root extracts of *Sesbania sesban*, and concluded that OAG, has significant spermicidal activity.

Leaves of *Ocimum sanctum* L. (Labiatae) have been reported to possess antifertility activity in both sexes of rat and in male mice. Ursolic acid, ($C_{30}H_{48}O_3$), a triterpene was identified as the major bioactive chemical from the leaves of this plant (Seth *et al.*, 1981; Ramachandran Nair *et al.*, 1982). Further studies indicate that ursolic acid has some significant antifertility activity in male rats (Rajasekaran *et al.*, 1988). In a review by

Singh *et al.* (2010), they reported that ursolic acid is one of the major constituents of *Ocimum sanctum* (Tulsi) and has been proven to have promising anti-fertility ability devoid of side effect. In males, Tulsi leaves reduce spermatogenesis by retarding sertoli cells activity (Ahmed *et al*., 2002; Prakash and Gupta, 2005). The leaves of *O. sanctnum* have been shown to possess anti-implantation activity in experimental albino rats, and ursolic acid is responsible for its anti-sterility property. Benzene extract of *O. sanctum* leaves in albino rats decreases sperm motility and the total sperm count and, also showed antiandrogenic properties. Ursolic acid has been shown to possess antiestrogenic effect, and is responsible for the arrest of spermatogenesis in males and inhibition of implantation in ovum of rats and mice (Pratibha and Laxmi, 2005; Raja, 2012). Akbarsha *et al.* (1998) demonstrated that treating Wistar albino male rat with ursolic acid leads to symplast formation in the spermatogenic clones and pathological changes in the clear cells of the epithelium of ductus epididymidis. The authors suggested a prospective application of ursolic acid in male antifertility/ contraception.

2.9.4 Phytoestrogenic properties of Pawpaw and Moringa

Evidence from studies using Pawpaw (*Carica papaya*) seeds suggested that they exhibit antifertility and abortifacient properties. It has been used as fertility control agent in some animal models and even on human beings (Joshi and Chinoy, 1996; Udoh and Kehinde, 1999; Lohiya *et al*., 2004; Raji *et al*., 2005, Krishna *et al*., 2008). Histological evaluation of gonadal tissue obtained from Nile tilapia, *Oreochromis niloticus*, fed Pawpaw (*Carica papaya*) seed powder at 122gP/kg diet (Ekanem and Okoronkwo, 2003) and 2.0g/kg diet (Jegede and Fagbenro, 2008). These studies indicated that pawpaw seeds appear to be an effective sterility inducing agent through the disintegration of gonadal cells, preventing testical and ovarian development inot spermatids and oocytes.

Ethanolic and aqueous extract of *Moringa oleifera* roots is reported to possess estrogenic, anti-estrogenic, progestational and antiprogestational activities in rats (Prakesh *et al*., 1985; Shukla *et al*., 1988). Aqueous extracts of the root and the bark of *M. oleifera* are effective in preventing implantation in rats (Shukla *et al*., 1989). According to Bose (2007), studies have shown that *M. oleifera* inhibits maintenance and growth of reproductive organs.

These studies suggest that contraceptive efficacy of dietary plant nutrients with antifertility or arbortifacient activity can be found to combat problems of tilapia precocious breeding in aquacultural ponds. This method of control, if proved to be effective could be easier to adopt by poor fish farmers, particularly in sub-Saharan Africa, since the plants this study will be using are available all year round in the tropics and subtropical regions.

2.9.5 Pawpaw (*Carica papaya*)

The Pawpaw tree belongs to a small family called Caricaceae, which has four genera (*Carica papaya*, *C. cauliflora*, *C. pubescens* and *C. quercifolia*) in the world. The pawpaw or papaya (*Carica papaya* L.) is the most economically important fruit in this family (Figure 2.13).

Figure 2.13 The Pawpaw (*Carica papaya*) plant with fruits.

The Pawpaw is considered native to southern Mexico and neighbouring Central America. It is currently cultivated in Florida, Hawaii, East Africa, South Africa, Sri-Lanka, India, Canary Islands, Malaysia and Australia. Pawpaw (*Carica papaya*) is now grown in all tropical and most subtropical countries, particularly sub-Saharan Africa and many sub-tropical regions of the world (Krishna *et al.,* 2008). The percentage composition of typical pawpaw is: seed (8.5 %), skin (12 %) and pulp (79.5 %).

Biochemical Composition

Pawpaw contains a broad spectrum of phytochemicals including polysaccharides, vitamins, minerals, enzymes, proteins, alkaloids, glycosides, fats and oils, lectins, saponins, flavonoids, sterols (Table 2.2). Pawpaw contains phytochemicals including alkaloids, anthraquinones, flavonoids, glycosides, reducing sugars, saponins and tannins. Biochemically, all parts of the pawpaw plant produce useful constituents including proteins and alkaloids with important industrial applications (i.e. beverage, food, medicinal and pharmaceuticals). Of all the phytochemicals, papain is particularly important one. It is a proteolytic enzyme that is produced in the milky latex of green, unripe papaya fruits (the ripe papaya fruit contains no latex or papain) and can be produced from dried papaya latex collected from the immature fruit. Papain is used extensively in industry; in-chill proofing beer, food and tanning, tenderising meat and cheese processing industries (Teng, 1973; Emeruwa, 1982; Brocklehurst and Salih1985; OECD, 2003).

Table 2.2 Chemical composition of various parts of Pawpaw plant (Krishna et al., 2008)

Parts	Constituents
Fruits	rotein, fat, fibre, carbohydrates, minerals (calcium, phosphorus, iron), vitamin C, thiamine, ɔoflavin, niacin, and carotene, amino acids, citric and malic acids (green fruits), volatile ɔmpounds(linalool, benzylisothiocynate, cis and trans 2, 6-dimethyl-3,6 epoxy-7 octen-2-), Alkaloid, α; carpaine, benzyl-β-D glucosside, 2-phenylethyl- β-D-glucoside, 4-hydroxy-henyl-2-ethyl- β-D-glucoside and four isomeric malonated benzyl- β-D-glycosides
Juice	N-butyric, n-hexane and n-octanoic acids, lipids; myristic, palmitic, stearic, linoleic, linolenic and *cis*-vaccenic and oleic acid
Seed	Fatty acids, crude protein, crude fibre, papaya oil Carpaine, benzylisothiocynate glucotropacolin, benzylthiourea, hentriacontane, β-Sitosterol, caricin, and an enzyme myrosin
Root	Carposide, and an enzyme myrosin
Leaves	Alkaloids carpain, pseudocarpain, and dehydrocarpaine I aan II, choline, carposide, vitamin C and E
Bark	β-Sitosterol, glucose, fructose, sucrose, galactose and xylitol
Latex	Proteolytic enzymes, papain and chemopapain, glutamine cyclotransferase, chymopapains A, B and C, peptisidase A and B and lysozymes

According to Teng (1973) benzyl isothiocyanate (BITC) is found in enzymic extracts from various plant families, including Caricaceae and Moringaceae (Passera and Spettoli, 1981) The pawpaw seed composition includes free amino acids and proteins (e.g. aspartic acid, glycine, glutamic acid, serine, threonine, valine, cystein, methionine) essential amino acids (Isoleucine, Leucine, Lysine, Threonine, Tryptophan, valine); sugars (sucrose, xylose, glucose, and fructose); fatty acids (Oleic acid-76%,Palmitic acid-15%, Stearic acid-5% and Linoleic acid- 3% acid) and minerals (Ca, Na, K and Mg). The authors suggested that pawpaw seed could be a potential source of protein and oil in poultry feed. Emeruwa (1982) reported that pawpaw contains papain, chymopapain, lysozyme, proteinase, thioglucosidase and a protein-like substance which showed a significant antibacterial activity against both gram-positive and gram-negative bacteria, some of which are found in wounds (*Staphylococcus aureus* and *Escherichia coli*). Wilson *et al.* (2002) reported the presence of BITC in crushed papaya seed, which is believed to have activity against helminthic intestinal parasites.

The seeds of pawpaw are reported to contain 24.3g protein, 25.3g fatty oil, 32.5g total carbohydrate, 17.0g crude fibre, 8.8g ash, 0.09g volatile oil, a glycoside, caricin, and the enzyme, myrosin, per 100g of seed. The fatty oil of the seeds contains 16.97% saturated acids (11.38% palmitic, 5.25% stearic, and 0.31% arachidic) and 78.63% unsaturated acids (76.5% oleic and 2.13% linoleic). With reversed-phase partition column

chromatography, the oil from the seeds of *Carica papaya* has been found to contain lauric, 0.4%; myristic, 0.4%; palmitic, 16.2%; stearic, 5.0%; arachidic, 0.9%; behenic, 1.6%; hexadecenoic, 0.8%; oleic, 74.3%; and linoleic, 0.4%. It has been concluded that papaya seed is a rich source of nutrients with high potential as a source of high oleic oil, and its full potential should be exploited (Badami and Daulatabad, 1966; Marfo *et al.,* 1986; Puangsri *et al*., 2005; Unuabonah *et al*., 2008). According to Patisaul and Jefferson (2010) the most common phytoestrogen found in most edible plants are flavonoids (flavonols, isoflavonoids, and isoflavans); coumestans (coumestrol); lignans; saponins and the triterpenes. Among these, saponins and flavonoids have been identified in the Pawpaw plant parts.

Nutritional Role in Human; Poultry; and Livestock

Economically, *Carica papaya* is the most important species within the Caricaceae, being cultivated widely for consumption as a fresh fruit and for use in drinks, jams candies and as dried and crystallised fruit. Green fruit, leaves and flowers are also used as a sourse of nutrition. Pawpaw is also a good source of calcium, and an excellent source of vitamins A and C (Villeges, 1997; Watson, 1997; Naksone and Pauli, 1998). The vitamin A and C content of one medium papaya approaches or exceeds the USDA minimum daily requirements for adults (OECD, 2003; 2010).

Pawpaw has the ability to tenderize meat, and as such meat is cooked with raw or green pawpaw to makes it tender and digestible. It is considered as a common man's fruit, available in the tropics all year round, which is reasonably priced and possess a high nutritive value. Pawpaw fruits is palatable while rich in antioxidant nutrients like carotene, vitamin C, vitamin B, flavonoids, folate, panthotenic acids and minerals such as potassium and magnesium. The fruit is also a good source of fibre, and is reported to promote the functions of cardiovascular system and provide protection against colon cancer (Krishna *et al*., 2008; OECD, 2005; 2010).

Pawpaw seed is readily available in the tropics because the fruits can be found all year round. After consumption of the fruit by humans, the seeds are thrown away and are regarded as a waste product. As the search for cheap sources of feed for livestock, poultry and aquaculture industries continues, the potential of pawpaw seed need to be evaluated. Passera and Spettoli (1981) suggested that pawpaw seed could be used in animal feeds, after establishing its nutritional importance. The dried skin of pawpaw fruit, which contain crude protein of 229g/kg, has been established as a potential source of dietary protein for broiler chickens and growing pullets (Fouzder *et al*., 1999; OECD, 2010). A feeding trial was conducted by Bolu *et al.* (2009), to investigate the effect of graded levels of Dried Pawpaw Seed (DPS) on growth performance, haematological parameters and carcass evaluation of chicken broilers over six weeks period at 0, 5, 10 and 15% DPS. The authors concluded that results from the study indicated that DPS can be included in broiler diet at 5% level.

Most pawpaw processing by-products are fed to buffalo, fish, and poultry. The by-product of pawpaw obtained after extraction of the fruit juice is called papaya pomace (PP). It contains peels and seeds from the fruit, and is known to have high protein content of 30.1%. Babu *et al.* (2003) fed buffalo with PP, and found

that the digestibility was 49.9% of dry matter and 51.4% of protein. Azevêdo *et al.,* (2011) established that PP has a higher energy value than maize, and thus could replace it in diets for growing cattle and pigs. The nutrients of major concern for buffalo are crude protein, crude fat (ether extractable), crude ash, carbohydrates, neutral detergent fibre (NDF), acid detergent fibre (ADF), calcium and phosphorus. The major nutrient considerations for fish feeds are apparent protein digestibility (APD) and amino acid levels in pawpaw leaf meal, however APD is not expected to be routinely measured in feed (Eusebio and Coloso, 2000; OECD, 2010).

Medicinal and Pharmacological Properties

Different parts of the plant are applied in the treatment of different human and veterinary diseases in various parts of the world. The medicinal or pharmacological properties of the Pawpaw plant is attributed to some major active ingredients which include, alkaloids, carpine, chymopapain and papain, a bactericidal aglycone of glucotropaeolin, benzyl isothiocyanate, a glycoside sinigrin, the enzyme myrosin, and carpasemine, glycosides, phenols, saponis and tannins (Akah *et al*., 1997; Wilson, 2002; Doughari *et al*., 2007; 2009). These biologically active phytochemicals have been reported to have various medicinal and pharmaceutical properties of which being antimicrobial; anthemintic, anti-amoebic, antimalarial and antifungal in nature (Krishna *et al*., 2008).

The Pawpaw fruit and seed extracts possesses bactericidal activity against *Bacillus cereus, Escherischia coli, Pseudomonas aeruginosa, Shigella flexneri* and *Staphylococcus aureus* (Emeruwa, 1982). It has, been documented that the pulverized seeds possess anti-parasitic activities against *Entamoeba histolytica*, and *Dirofilaria immitis* infections and are therefore used in ethnoveterinary practices (Lans *et al*., 2000). Adu *et al.* (2009) reported that papain from latex Pawpaw possesses pharmacotherapeutic properties against intestinal nematodes of poultry, and that it could be used as a deworming agent in the poultry industry.

Extracts are reported to have sedative and muscle relaxant properties and have been shown to have an effect on vascular contraction using a canine carotid artery in *in vitro* (Wilson, 2002). Molliscidal activity of the seed, and lyophilized latex powder, of papaya against freshwater snail *Lymnaea acuminate* has also been established and the pawpaw extract was not toxic to the fish *Colisa fasciatus* which shares the same habitat with the snail (Jaiswal and Singh, 2008).

Ezike *et al.* (2009) evaluated the effects of aqueous and methanol extracts, from unripe whole *Carica papaya* fruit, which proved to protect gastric mucosa against indomethacin and ethanol induced gastric ulcer in rats, and also inhibited gastrointestinal propulsion. The results show that unripe *C. papaya* fruit has potentials in the management of ulcers and justifies the use of the unripe fruit in traditional medicine practice to manage ulcers. Adeneye and Olaguiya (2009) demonstrated the potential of *C. papaya* in management of hypoglycemia, hypolipidemia and cardioprotectiveness in rats, confirming its use in traditional herbal treatment of suspected type 2 diabetes in Nigeria. Nwangwa (2012) evaluated the effect of aquoues extract of different concentrations of ripe *C. papaya* seed on the liver function enzymes on adult Wister rats for 4-

weeks. The author observed that the results showed a positive hepatoprotective effect, and concluded that *C. papaya* could be used as a supplement in the management of liver damage.

Effect on Reproduction

Ekanem and Okoronkwo (2003), Jegede and Fagbenro (2008), and Abbas and Abbas (2011) all mentioned that Pawpaw seeds contain active ingredients such as caricacin, an enzyme carpasemine, a plant growth inhibitor, and oleanolic glycoside. These authors adduced that Das (1980) contended that oleanolic glycoside is an active component of pawpaw seed, which had been found to cause sterility in male rats. According to Das (1980), citing Fransworth (1975), the active principle responsible for the antiimplantation effect of papaya seed in female rats, might be 5-hydroxytryptamine, but it remains to be seen whether the same compound is responsible for the antifertility activity in male rats.

The critical question is whether 5-hydroxytryptamine is the same as oleanolic glycoside, the active component in pawpaw seed that has been cited by Ekanem and Okoronkwo (2003), Jegede and Fagbenro (2008) and Abbas and Abbas (2011) as the recorded component in Pawpaw seed mentioned by Das (1980) causes sterility in male rats.

5-hydroxytryptamine (5-HT) is also known as serotonin, a hormone and neurotransmitter. According to Green (2006) and Filip and Bader (2009) serotonin (5-HT) is involved in numerous physiological and behavioral disorders, such as anxiety, major depression, schizophrenia, autism, obesity and drug addiction, acting as an important neurotransmitter in the central nervous system

The 5-HT1A receptors (one of the 5-HT_1 receptor family) are involved in motor behaviour (activation evokes flat body posture, forepaw treading, tail flick, lower lip retraction and locomotor activation), copulatory behavior (activation reduces penile reflexes, frequency and length of intromision, and increases latency of ejaculation), pain perception (activation evokes analgesia) and emotional behaviour (activation induces anxiolysis) (Kennett, 1998; Lanfumey and Haamon, 2004).

From the above it might mean 5-hydroxytryptamine is not the same as oleanolic glycoside, mentioned by Ekanem and Okoronkwo (2003), Jegede and Fagbenro (2008) and Abbas and Abbas (2011) all citing Das (1980). Studies should be conducted to ascertain the nature of these two compounds and their relatedness.

Kumar *et al.* (2012), metioned oleanolic acid-3-glucoside as one of the phytochemicals isolated from a list of 577 plant species (which includes Pawpaw and Moringa) that have shown 100% antifertility activity in animal models. It is possible that oleanolic acid-3-glucoside could be the active component of Pawpaw seed mentioned by Das (1980) and [cited by Ekanem and Okoronkwo (2003), Jegede and Fagbenro (2008) and Abbas and Abbas (2011)] as the antifertility or sterility agent in Pawpaw seed. Pillai *et al.* (1982) has also reported the antifertility activity of oleanolic acid-3β-glucoside.

Evidence from several studies on Pawpaw, *Carica papaya* fruits (epicarp, endocarp, seeds and leaves) suggests that it contains antifertility properties. The seeds in particular have been the subject of significant evaluation using animal models and even testing on human sperm. Many of these studies have been conducted in India and Southeast Asia, where there is considerable interest in developing safe and effective oral male contraceptive (Lohiya and Goyal, 1992; Lohiya *et al.*, 1999; Lohiya *et al.*, 2000; Raji *et al.*, 2005).

The crude extracts of *Carica papaya* seeds have been reported to cause variable responses in contraceptive efficacy in mice, rats and rabbits. Among the various crude extracts, aqueous, methanol, ethyl acetate, benzene and chloroform have been tested for the contraceptive efficacy, orally (e.g. 5mg/animal/day), for a number of days, usually between 60 and 150 days.

Different extracts from aqueous, benzene, chloroform and methanol tested in rats and mice have all reported suppression of spermatogenesis following the administration of the pawpaw seed extract (Chinoy *et al.,* 1994; Joshi and Chinoy, 1996; Lohiya *et al.*, 2000; Raji *et al.*, 2005, Goyal *et al.*, 2010). These extracts were also not found to cause impairment in libido nor do they display any toxicity (Lohiya *et al.*, 2006; Goyal *et al.*, 2010). Udoh and Kehinde (1999) also reported that Pawpaw (*Carica papaya*) seed, administered orally to male albino rats, effectively controlled their reproduction. Aqueous and chloroform extracts appeared to be species specific, for they have shown 100% efficacy in mice and rats (Lohiya and Goyal, 1992; Lohiya *et al.*, 1994; Pathak *et al.*, 2000), but were ineffective in rabbits (Lohiya *et al.*, 2000).

In all these studies, the inhibition of fertility coincided with reduced motility of caudal epididymal spermatozoa, reduced sperm count in the testis and cauda epididymis, and changes in the morphology of the spermatozoa, without affecting the body weight, accessory reproductive organs weight, haematology and serum biochemistry values, suggesting safety of the drug. The effects were completely reversible following 60 days withdrawal of the treatment (Pathak *et al.*, 2000, Verma *et al.*, 2006). A complete loss of fertility has also been reported in male rabbits and monkeys fed an extract of papaya seeds. Lohiya *et al.* (2002) suggested that ingestion of papaya seeds may adversely affect the fertility of human males or other male mammals.

These studies suggest a similar effect would be seen in male tilapia. Das (1980) reported that Pawpaw seeds contain oleanolic glycoside, an active ingredient which was found to cause sterility in male rats. Histological observation on Nile Tilapia, *Oreochromis niloticus*, fed with Pawpaw (*Carica papaya*) seed powder showed that Pawpaw seed may be an effective sterility - inducing agent as it caused disintegration of gonadal cells and testes and ovaries (Ekanem and Okoronkwo, 2003; Jegede and Fagbenro, 2008; Abbas and Abbas, 2011).

It has been reported in both Asian and African folk medicine that the latex of pawpaw is employed as an abortificant. Adebiyi *et al.*, (2002) indicated that consumption of unripe pawpaw, containing high concentration of the latex, could be unsafe during pregnancy, but that of ripe pawpaw pose no risk. In a study using 80% ethanol extract of pawpaw seed, Adebiyi *et al.* (2003), established that benzyl

isothiocyanate (BITC) the main bioactive and anthelmintic compound in different extracts of papaya seeds induced functional and morphological derangement of isolated uterus. These authors concluded that at high concentrations, ethanol extract is capable of causing irreversible uterine tocolysis probably due to the damaging effect of BITC (its chief phytochemical) on the myometrium. They suggest that unripe papaya fruit may induce miscarriage in susceptible pregnant human females since papaya is believed to have abortifacient properties (induces miscarriage during pregnancy) or teratogenic properties (causes malformations of the foetus).

Mansurah *et al.* (2009) performed a study to determine the effect of *Carica papaya* seeds used as a condiment, on pre-implantation embryo development in female Wistar rats. They reported no significant difference in the number of *corpora lutea* in treatment groups. The study concluded that the unfermented seed extract resulted in higher pre-implantation losses, indicating contraceptive characteristics. The fermented extract had no such effect and may be considered safe for human consumption.

2.9.6 Moringa (*Moringa oleifera*)

Moringa oleifera is a small to middle sized, a fast growing, perennial tree which can reach a maximum height of 7-12 m and reaches a diameter of 20-40 cm (Figure 2.14). According to Bennett *et al.* (2003), *Moringa* is a monogeneric family, the Moringaceae, which is divided into 10 species, all are xerophytic with *M oleifera* being the most commercially important.

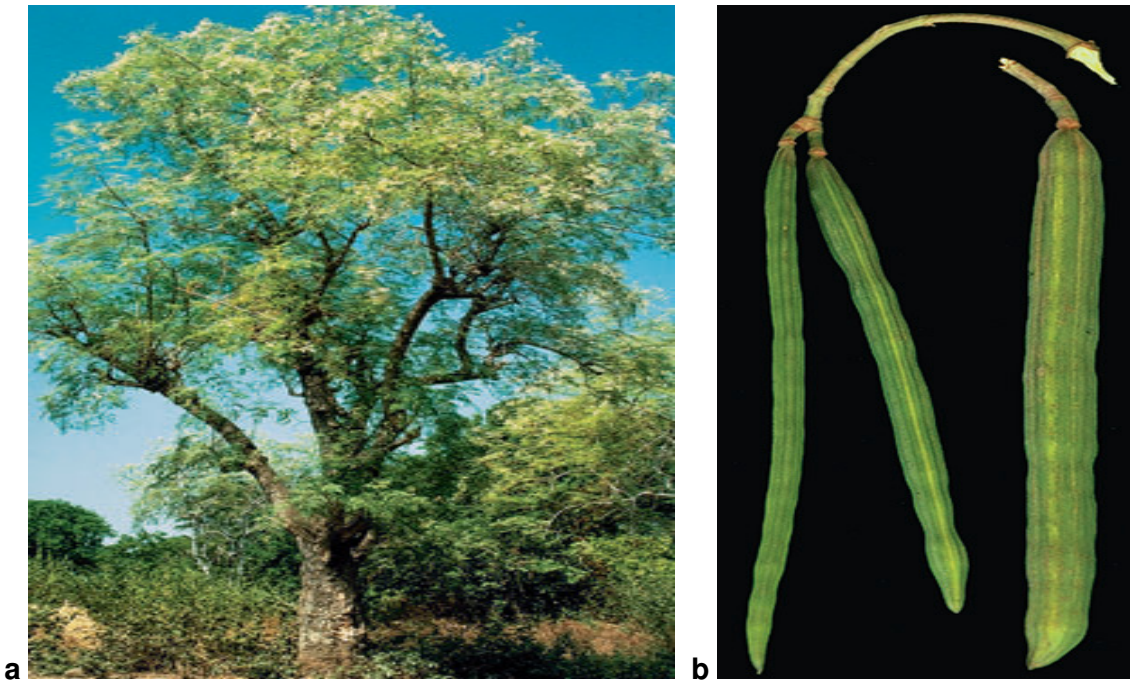


Figure.2.14 The *Moringa oleifera* tree a) a full grown tree b) fruits. (Adapted from Roloff *et al*., 2009]

The Moringaceae, is within the order Capparales (glucosinolate – containing species), and on the basis of molecular analysis, it has a closer link to the Caricaceae (e.g. *Carica papaya*). These glucosinolate containing species were utilized by the ancient Egyptians, Greeks and Romans for medicinal puposes (Johnson, 2002).

Moringa oleifera is indigenous to parts of India, Pakistan, Bangladesh and Afghanistan and today is widely cultivated in the tropical regions of Asia, including India, the Philippines, parts of West, East and Southern Africa, Latin America, the Caribbean, Florida and the Pacific Islands. It is a perennial softwood tree with timber of low quality and has used in traditional medicinal and other industrial uses. All parts of the Moringa tree are been consumed by humans with the secondary metabolites having the potential to affect health (Fugile, 1999; Fahey, 2005; Anwar *et al*., 2007; Pandey *et al*., 2011).

According to Fuglie (1999), Moringa has many uses including: animal forage (leaves and treated seed-cake), biogas (from leaves), domestic cleaning agent (crushed leaves), blue dye (wood), alley cropping (biomass production), fencing (living trees), fertilizer (seed-cake), foliar nutrient (juice expressed from the leaves), green manure (from leaves), gum (from tree trunks), honey- and sugar cane juice-clarifier (powdered seeds), honey (flower nectar), medicine (all plant parts), ornamental plantings, biopesticide (soil incorporation of leaves to prevent seedling damping off), pulp (wood), rope (bark), tannin for tanning hides (bark and gum), water purification (powdered seeds). Its versatility as a medicine, food, nutraceutical and water purifying potential, has giving this plant a high economic value.

Biochemical Composition

An examination of the phytochemicals of Moringa species affords the opportunity to examine a range of fairly unique compounds (Table 2.3).

Biochemical analysis has revealed that the family Moringaceae is rich in compounds containing the simple sugar, rhamnose, and it is rich in a fairly unique group of compounds called glucosinolates and isothiocyanates. For example, specific components of Moringa preparations that have been reported to have hypotensive, anticancer, and antibacterial activity include niazimicin, pterygospermin, benzyl isothiocyanate, 4-(4′-*O*-acetyl-a-L-rhamnopyranosyloxy)benzyl isothiocyanate, 4-(a-L-rhamnopyranosyloxy) benzyl isothiocyanate, and 4-(a-L-rhamnopyranosyloxy)benzyl glucosinolate, with many of these compounds unique to the Moringa family (Table 2.3). It is also rich in a number of vitamins and minerals as well as other more commonly recognized phytochemicals such as the carotenoids (including-carotene or pro-vitamin A) (Faizi *et al.,* 1994; 1998; Bennet *et al*., 2003; Anwar *et al*., 2007).

Table 2.3 Phytochemical constituents isolated from *Moringa oleifera* Lam**.** (Adapted from Bennet, *et al*., 2003; Anwar *et al*., 2007)

Parts	Phytochemical constituents
Roots	4-(α-L-rhamnopyranosyloxy)-benzylglucosinolate and benzylglucosinolate
Stem	4-hydroxymellein, vanillin, β-sitosterone, octacosanic acid and β-sitosterol
Bark	4-(α-L-rhamnopyranosyloxy)-benzylglucosinolate 10
Whole gum exudates	L-arabinose, D-galactose, D-glucuronic acid, L-rhamnose, D-mannose, D-xylose and leucoanthocyanin 12-13
Leaves	Glycoside niazirin, niazirinin and three mustard oil glycosides, 4-[4'-O-acetyl- α -L-rhamnosyloxy) benzyl] isothiocyanate, niaziminin A and B; quercetin, kaempferol
Mature flowers	D-mannose, D-glucose, protein, ascorbic acid, polysaccharide 16
Whole pods	Nitriles, isothiocyanate, thiocarbanates, 0-[2'-hydroxy-3'-(2''-heptenyloxy)]-propylundecanoate, 0-ethyl-4-[(α -1-rhamnosyloxy)-benzyl] carbamate, methyl-p-hydroxybenzoate and β-sitosterol 14-15
Mature seeds	Crude protein, Crude fat, carbohydrate, methionine, cysteine, 4-(α-L-rhamnopyranosyloxy)-benzylglucosinolate, benzylglucosinolate, moringyne, mono-palmitic and di-oleic triglyceride 10
Seed oil	Vitamin A, beta carotene, precursor of Vitamin A 17-18

As indicated, the most common phytoestrogens that are found in most edible plants include flavonoids (flavonols, isoflavonoids, and isoflavans); coumestans (coumestrol); lignans; saponins and the triterpenes (Patisaul and Jefferson, 2010); and the reported phytoestrogens identified in Moringa plant parts include saponins and flavonoids (quercetin and kaempferol) (Table 2.3).

Nutritional Role in Humans; Poultry, and Llivestock

As an important tropical food crop *Moringa oleifera* Lam is grown for various purposes in sub-Saharan Africa (especially Central, East and West Africa; and tropical southern Africa) for human consumption (i.e. vegetable - leaves, flower, buds, roasted seed kernels, spices, as a source of cooking and cosmetic oil), herbal medicines, as a male aphrodisiac (seed kernels), water purification, livestock forage and animal feed (leaves, stem, and seed meal processed to remove the coagulating proteins), a source of tannins and dyes (bark) and fuel (seed husks and wood) (Makker and Becker 1997; Foidl *et al*., 2001; Kumar *et al.,* 2010; Sánchez-Machado, 2010; Dubey *et al.,* 2013).

In most Sub-Saharan countries as well as India, Pakistan, Philippines, and Hawaii most parts of the plant, including the leaves, fruit, flowers and immature pods, of this tree is utilized. For example, seeds are eaten green, roasted, powdered and steeped for tea or used in curries (Anwar and Bhanger, 2003; Anwar *et al.*, 2005). In a study to evaluate the nutritive quality of *Moringa oleifera* edible parts, Sanchez-Machado *et al.* (2010), concluded that it could be successfully utilized as food for human consumption. The leaves and flowers are a protein source with an adequate profile of amino acids and ash, while the immature pods show a high content of dietary fiber and low lipid content. Unsaturated and essential fatty acids are present in all samples. The collection and consumption of all the parts of the plant could have financial and social benefits for the population of countries where this tree is being cultivated. The flowers are a rich source of amino acids, D-glucose, sucrose, traces of alkaloids, wax, quercetin and kaempferat with the ash containing high levels of potassium and calcium.

Moringa leaves have been reported to be a rich source of β-carotene, protein, iron, vitamins A, B and C, calcium, iron, phosphorus, copper, and potassium and act as a good source of natural antioxidants. It also enhances the shelf-life of fat containing foods due to the presence of various types of antioxidant compounds such as ascorbic acid, flavonoids, phenolics and carotenoids. The leaves contain various types of antioxidant compounds including ascorbic acid, flavonoids, carotenoids and phenolics. They have also been reported to contain some flavonoid pigments such as alkaloids, kaempherol, estrogenic substances, β-sitosterol, rhamnetin, isoquercitrin and kaempferitrin. The high concentrations of ascorbic acid, α-tocopherol, riboflavin, nicotinic acid, folic acid, pyridoxine, and in particular essential amino acids such as methionine, cystine, tryptophan and lysine present in *Moringa* leaves and pods make it a balanced dietary supplement (Makkar and Becker, 1996; Anwar *et al.*, 2005; Dillard and German, 2000; Siddhuraju and Becker, 2003; Bennett *et al.*, 2003; Anwar *et al.*, 2007; Kumar *et al.,* 2010). In the Philippines it is utilized to enhance milk production in lactating woman and is sometimes prescribed for anemia (Estrella *et al.*, 2000; Siddhuraju and Becker, 2003; Dubey *et al.,* 2013).

Studies on the leaves of Moringa as part of poultry feed have been conducted, which showed that the leaves contained appreciable amounts of poultry nutritional requirements. In one such study Moringa oleifera leaf meal (MOLM) as a protein source for chicken layers was evaluated. The results showed that the feed and dry matter intake demonstrated MOLM as palatable and highly preferred by chickens. Also a high performance in egg production was observed in comparison with other leaf meals from an earlier study. The study also established that Moringa leaf meal has high protein content, relatively low fibre and higher mineral contents, and thus could be used as a substitute for sunflower seed meal. The authors concluded that *Moringa oleifera* has a potential in poultry feeding and that it could be given as a sole plant source. Also in areas where MOLM can be obtained for free and quality of eggs fetch higher premium, complete substitution (20%) with MOLM was highly recommended. Therefore, Moringa leaves are highly recommended as a useful feed supplement and as medicine in poultry to improve health and growth performance (Kakenge *et al.*, 2007; Ogbe and Affiku, 2011). The anti-nutritional factors (Richter *et al.*, 2003) reported to be present in this plant parts could be reduced through adequate processing by boiling in hot water during aqueous extraction.

Moringa grows in all types of soil, from acid to alkaline and at altitudes from sea level to 1800 m. It is drought tolerant and will grow even during the 6 months of the dry season and thus does well in the tropics. Reports from evaluation of Moringa as forage for small ruminants indicate that all parts of the plant have considerable potentials as supplements to low quality diet that can be used in the feeding of goats, sheep and cows (Mandal, 1997; Sánchez *et al*., 2006). Aregheore (2002) evaluated the nutritive value and digestibility of *Moringa oleifera*–batiki grass (*Ischaemum aristatum* var. *indicum*) mixtures offered to the growing goats in the humid tropical environment of Western Samoa. The author found that the apparent nutrient digestibility coefficients in all the diets were above 50% digestibility level; and thus concluded that *Moringa oleifera* at 20 and 50% levels of total daily forage allowance could be used as a cheap protein supplement in batiki grass based diets for goats. Ben Salem and Makker (2009) found that defatted Moringa seed used as feed additive to hay, improved the growth rate of lambs. It has also been established that inclusion of Moringa as a protein supplement in cow diets improved dry matter intake and digestibility of the diet, and increased milk production, and did not affect milk composition. Moringa leaf meal is a potential source of protein to supplement poor-quality forage such as Elephant grass. It can successfully replace commercial concentrate constituents for dairy cows as long as the substitution is isocaloric and isoproteinic (Blache *et al*., 2008; Mendieta-Araica *et al*., 2011).

Preliminary studies on nutritional quality of raw Moringa leaves as partial feed for fish indicate that up to 10 % -30 % inclusion of the material can be recommended for Nile tilapia. In view of the favourable amino acid profile of Moringa leaves and their wide and ready availability throughout the tropics and subtropics, the authors recommended that Moringa can be considered as a potential feed component with high nutritive value for fish (Afuang *et al*., 1997; Richter *et al*., 2003; Makkar *et al.,* 2007).

Medicinal and Pharmacological Properties

Several studies and reports show that all parts of the plant *Moringa oleifera* and their extracts are rich sources of a number of vitamins and minerals (Table 2.3), which may contribute significantly to its antioxidant, therapeutic, and nutritional value. The leaves, seeds and green immature pods serve as a good source of natural antioxidants due to the presence of various types of antioxidant compounds such as ascorbic acid, flavonoids, phenolics and carotenoids. The high concentrations of ascorbic acid, iron, calcium, phosphorus, copper, vitamins A, B and C, α-tocopherol, riboflavin, nicotinic acid, folic acid, pyridoxine, β-carotene, protein, and in particular essential amino acids such as methionine, cystine, tryptophan and lysine present in Moringa leaves and pods make it a virtually ideal dietary supplement (Makkar and Becker, 1996; Anwar *et al*., 2007).

Together with its high nutritional value a range of medicinal properties such as cardiac and circulatory stimulants, possess antitumor, antipyretic, antiepileptic, antiinflammatory, antiulcer, antispasmodic, antihypertensive, antioxidant, antidiabetic, hepatoprotective, cholesterol lowering, diuretic, antibacterial and antifungal activities (Kumar *et al*., 2010; Dubey et al., 2013). Some parts of the plant has been reported to contain important minerals, and are a good source of protein, vitamins, β -carotene, amino acids and various

phenolics (Kumar et al., 2010). Other parts of this plant such as the leaves, roots, seed, bark, fruit, flowers and immature pods provide antibiotic, antitrypanosomal, antispasmodic, antiulcer, anti-inflammatory, hypotensive, hypocholesterolemic, and hypoglycemic activities (Kumar *et al.,* 2010; Dubey *et al*., 2013). Moringa is also used for the treatment of different inflammation and infectious diseases along with cardiovascular, gastrointestinal, hematological and hepatorenal disorders (Anwar *et al.,* 2007; Kumar *at al,* 2010; Flora and Pachauri, 2011; Mehta *et al*., 2011).

Some of its numerous medicinal and pharmaceutical properties include:

(i) Protection against oxidative DNA damage

According to Flora and Pachauri (2011) oxidative stress adversely affects all cell types that contributes to pathological conditions, which justifying the use of antioxidants in treating various chronic diseases. The presence of phenolics and flavonoid groups such as quercetin and kaempferol, enable moringa to function a natural antioxidant (Siddhuraju and Becker, 2003), inhibiting oxidative DNA damage (Singh *et al*., 2009).

(ii) Anti-inflammatory activity

Inflammation is an underlying cause for various diseases, or their manifestation. It is reported that Moringa extracts provide protection against several inflammatory pathological conditions (Siddhuraju and Becker, 2003; Anwar *et al*., 2007).

(iii) Antitumor and Cancer prevention

Chemoprevention of cancer involves the use of natural or synthetic agents to inhibit, delay, or reverse the development of cancer. *Moringa* leaves have been shown to display significant antitumor activity (Anwar *et al*., 2007).

(iv) Antimicrobial activity

Moringa extracts display antimicrobial activity in a number of bacteriological studies through benzyl isothiocyanate as active ingredient (Oliver-Bever, 1983; Sutherland *et al*., 1990; Suarez *et al.,* 2003; Asare et al., 2012)

Effect on Reproduction

Moringa oleifera Lam has been mentioned in the literature for its antifertility effect. Shukla *et al.* (1988) cited that Soejarto (1978) categorized it as an antifertility plant. In a review by Anwar *et al.* (2007), the parts of the plant listed as having or showing abortifacent or antifertility property include root, gum and flowers, and exclud stem bark, leaves and seeds. However, some earlier studies on antifertility profile of Moringa species had included leaves (Nath *et al.,* 1992) and stem bark (Skukla *et al*., 1988; 1989). Sethi *et al.* (1988) have noted that contraceptive potency of many plants varied considerably with respect to season, temperature and place of collection.

It has, been reported that different fractions of Moringa manifest significant antifertility activity in rats. For instance, the root extracts from water and ethanol have been confirmed to show strong abortifacient

properties including anti-progestational and anti-estrogenic activities. Aqueous extracts of the root and the bark of *M. oleifera* are effective in preventing implantation in rats ((Prakesh *et al*., 1985; Shukla *et al*., 1988; Sethi *et al*., 1988; Shukla *et al*., 1989; Kumar *et al*., 2010; Kumar *et al*., 2012).

Nath *et al.* (1992) studied 17 indigenous plants for abortifacient activity in rats, using 90% ethanol and water as the solvents for extraction. The study looked at the pregnancy and foetal development within 10 days after artificial fertilization and orally given the extracts. The results indicated that 17mg/kg of *Moringa oleifera* or *Adhatoda vasica* leaves extract showed 100% abortifacient activity.

Bose (2007) reported that *Moringa oleifera* has the capacity to prevent reproductive growth and maintenance. This author intimated that in West Bengal, India, the rural women use Moringa roots to prevent pregnancy because, it can put the reproductive system in a dormant state.

The nutritional quality of *M. oleifera* leaves have been use in attempt as a replacement for protein in *Oreochromis niloticus diet.* It has been recommended that 30% fishmeal in the fish diet can be substituted with Moringa leaves containing 25% crude protein (Afuang *et al*., 1997; Ritcher *et al*., 2003; Dongmeza *et al.,* 2006; Makker *et al.,* 2007). Unlike Pawpaw, no relevant literature could be found on attempts to elicit any form of antifertility activity in a cultured fish, including tilapia using Moringa extracts.

Other Uses - Coagulant/Flocculant

The production of potable water involves coagulant use at a flocculation/coagulation stage to remove turbidity in the form of suspended and colloidal material. According to Mangale *et al.* (2012) the seed of *Moringa oleifera* is used for the purification of drinking water. It has been established that the seed contains certain absorbing properties that allow them to floccutate or coagulate particles. It has the capacity to maintain, within acceptable levels, certain physico-chemical parameters of water such as turbidity, total hardness, fluorine and chlorine content, alkalinity and acidity, thereby making it fit for human consumption. Another cleaning property is that it contains microbicides, therefore, it is able to reduce bacteria load of water. The dry, crushed seed and seed pressed cake, contain polypeptides, that have the ability to serve as the natural coagulants for water treatment given it unique properties (Ndabigengesere *et al*., 1995; Diez *et al*., 2002; Santos *et al*., 2005). Lecitin has been found as the flocculent protein, found in the seed cake after oil extraction, which allows for a double valorisation of the seeds. Moreover, extracting the oil improves the flocculation efficiency, as when the whole seed powder is used its fatty nature causes flotation and filter clogging (Okuda *et al*., 1999; Santos *et al*., 2005; 2009).

Foidl (2003) reported that *M. oleifera* seeds have been used in treating water from the Nile River over the years in Egypt and Sudan for human consuption. Treating water with Moringa play two main roles (i) the antimicrobial activity enables it to reduce microbial load to acceptable level, and (ii) possess a coagulant protein with a positive charge capable of attracting the negatively charged particles in water such as bacteria, silt, clay and toxins.

Okuda *et al.* (2001) contend that natural biodegradable coagulants offer advantages to inorganic or synthetic organic polymers, which are associated with human pathological processes. Aluminium sulphate, usually used to clear turbid water, is dangerous to use at over 220 mg/l according to WHO. When turbidity is too high, which is often the case in tropical countries, a pre-treatment by sedimentation is necessary. By contrast, Moringa is especially efficient to treat highly turbid water, and high doses are not toxic. The use of aluminium sulphate is also more and more criticised because of the residues that are found in drinking water. Studies showed that soluble aluminium is toxic to fishes, crustaceans, molluscs and certain insects (Sauveur, 2001).

Moringa oleifera therefore, has potential in water treatment. Advantages observed by introducing the *Moringa oleifera* seeds as primary coagulant and coagulant aid has potential for its use in coagulation of turbid surface waters with high removal rates ranging from 97–99.9% of the impurities in water (Doerr, 2005; Liew *et al*., 2006; Lea, 2010; Santos *et al.,* 2012). Vieira *et al.,* (2010) reported that *Moringa oleifera* pods have been employed as an inexpensive and effective adsorbent for the removal of organics, and a coagulant for water treatment. It was also established that Moringa pods are non-toxic natural organic polymer with a removal efficiencies of up to 98%, for both colour and turbidity. The authors maintained that their results showed Moringa seed with strong adsorption power under a pH range between 5 and 8, with the potential to be used in the dairy industry wastewater treatment in an efficient way and with low cost.

2.10 Conclusion

Tilapia production plays an important role in global food security, in particular amongst rural communities of developing countries. Tilapia culture is negatively affected by the onset of early maturity followed by uncontrolled reproduction, overpopulation and retarded growth. Various techniques have been developed in an effort to control reproduction of tilapia in the culture system. Some of the more successful commercial techniques such as hormonal sex reversal and the use of monosex populations are not suitable for use by rural small farmers due to its complexity and difficulty to obtain the controlled substances (steroid hormones). The use of traditional phytoestrogenic compounds, readily available in most of tropical and Sub-Saharan Africa, such as pawpaw and Moringa, could provide an alternative and accessible method to control unwanted reproduction. The phytoestrogenic properties of pawpaw and Moringa and their effect as endocrine disruptors are discussed in view of its potential use to inhibit reproduction in small scale tilapia farming systems.

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Chapter 3

General Materials and Methods

The study consisted of three experiments that were, conducted between October 2010 and November 2011. The respective experiments investigated the potential of biochemical plant components, as endocrine disrupters, to suppress gonadal development and function in Mozambique tilapia (*Oreochromis mossambicus*).

3.1 The experimental location and facilities

The experimental unit was a recirculating aquaria system (RAS) consisting of 72-glass tanks (Plate 3.1). It was built inside a greenhouse, which is part of the Division of Aquaculture's (DA) facilities at Welgevallen Experimental Farm, Faculty of AgriSciences, located on a geographical position system (GPS), coordinates 33°56' 33.95" S and 18°51'56.15 "E, Stellenbosch University (SU), Stellenbosch, South Africa.

The experimental facilities consisted of a recirculating aquaria system that housed a total of 72 glass aquaria. The RAS consisted of two platforms; each platform had six racks, and each rack had two levels, with three tanks per level (Plate 3.1). Each tank had a volume of 121L (dimensions 57cm x 53cm x 40cm).

Each platform was supplied with controlled water reticulated system, a water filtration system, an aeration system, a water pump as well as a heating system. The filtration system consisted of a mechanical and biological filter and a sump (Refer to Appendix II for more details on the design of the respective components of the RAS).

Each platform consisting of 36 tanks had a separate filtration, heating and aeration system providing a constant flow of water (13.6 ± 1.9 L/seconds) and air (7.74mg/L/seconds) to each tank (Plate 3.1b & f). The outflow passed through a mechanical and biological filtration system to ensure that optimum growing conditions (pH 7-8, DO 7.0-8.2mg/L) were maintained throughout the study. Water temperature was maintained at 28 ± 2°C through the use of 1000kW heating element positioned in the sump section of the biofilter unit (Plate 3.1b).

Air supply to the tanks was provided by a 1.1kW blower (FPZ effepizeta, srl, Model SCL V4, Incorezzo, Milano, Italy) distributing compressed air via a flexible 5mm tube with an air stone at the end (Plate 3.1b and f). Dissolved oxygen levels were maintained above 80% saturation ranging from 7.4–8.04 mg/L. The pH of the water was maintained within 7.5–8.0. Basic water quality parameters such as temperature (°C), dissolved oxygen (in mg/L), degree of saturation (%), and pH were monitored daily using a HACH, HQ 40-d Multi instrument, with HACH LDO probe.

Each aquarium was cleaned daily throughout the duration of the study. The mechanical filter (a plastic basket filled with Japanese mat, aquastones and foam) was cleaned every second day by removing the

packaging and washing off all the solid and trapped materials. The larger tank units were cleaned on a weekly basis, with 10% of the volume being replaced and 1 kg common salt added to the system.

Plate 3.1 The recirculating aquaria system used during the study, incorporating 72-Glass Tanks showing:

(a) Line 1 of Platform I; (b) Line 2 of Platform I on left & Line 1of Platform II on right; (c) Line 2 of Platform II; (d) Water pumps & mechanical filter (e) Edge of the system of both Platforms; and (f) Top of the system – showing the inlet and airflow tubes into the tanks.

Fish were fed *ad libitum* (Baker, 1984) 3 to 5 times a day, depending on the conditions (such as active swimming) in the tanks. Also during the weekly cleaning of the mechanical filter, the fish were fed 2 times, due to disturbance of the water, addition of new or fresh water and disinfection by running salt through the whole experimental unit.

3.2 Experimental Layout and Design

Three experiments were conducted between October 2010 and November 2011 (Table 3.1) during three different stages of gonadal development in Mozambique tilapia (*Oreochromis mossambicus*), namely:

a) Experiment I: fish with sexually matured gonads
b) Experiment II: fish with sexually immature or maturing gonads
c) Experiment III: fry stage with undifferentiated gonads

Table 3.1 Experimental stages and timelines, conducted between October 2010 and November 2011.

Experimental details	Period	Size, Age Group
Ia: The effect of Pawpaw (*C. papaya*) and Moringa (*M. oleifera*) seed powder on the gonadal activity of sexually mature Mozambique tilapia (*O. mossambicus*)	October 2010 to December 2010	Sexually matured, Weight 20-45g, 3-4 months of age
Ib: The potential synergistic influence of Pawpaw (*C. papaya*), Moringa (*M. oleifera*) seed powder, and 17α-methyltestosterone, on the gonadal activity of sexually mature Mozambique tilapia (*O. mossambicus*)	October 2010 to December 2010	Sexually matured, Weight 20-45g, 3-4 months of age
II: The influence of Pawpaw (*C. papaya*) and Moringa (*M. oleifera*) seed powder on the gonadal development of sexually immature Mozambique tilapia (*O. mossambicus*) fingerlings.	April 2011 to June 2011	Sexually immature, Weight 2-8g, 1-2 months of age
III: The influence of Pawpaw (*C. papaya*) and Moringa (*M. oleifera*) seed powder on undifferentiated Mozambique tilapia (*O. mossambicus*) fry	July 2011 to November 2011	Undifferentiated fry Weight 0.001mg; 9-12 day post hatch

3.2.1 Experiment I.a: The effect of Pawpaw and Moringa treatments on the gonadal activity of sexually mature Mozambique tilapia (*O. mossambicus*)

The experimental design was that of 11 x 5 factorial design, made up of 11 treatments with five repeats per treatment. Five males and females were randomly allocated to each of the 55 tanks, with five tanks assigned as a repeats to each treatment. The details of the respective treatments are presented in Table 3.4.

3.2.2 Experiment I.b: The combined effect of Pawpaw and Moringa on the gonadal activity of sexually mature Mozambique tilapia (*O. mossambicus*)

The experimental design was that of 4 x 5 factorial design, made up of four treatments with five repeats per treatment. Five males and females were randomly allocated to each of the 20 tanks, with five tanks assigned as repeatitions to each treatment. The details of the respective treatments are presented in Table 3.5. Experiment Ia was conducted parallel to Experiment Ib, using the same Control group as reference.

3.2.3 Experiment II: The effect of Pawpaw and Moringa treatments on the gonadal activity of sexually immature Mozambique tilapia (*O. mossambicus*)

The experimental design was that of 11 x 5 factorial design, made up of 11 treatments with five repeats per treatment. Five males and females were randomly allocated to each of the 55 tanks, with five tanks assigned as repeats to each treatment. The details of the respective treatments are presented in Table 3.6.

3.3.4 Experiment III: The effect of Pawpaw and Moringa treatments on the gonadal differentiation of Mozambique tilapia fry (*O. mossambicus*)

The Pawpaw and Moringa treatments were conducted in two consecutive experiments. The experimental design for each treatment was that of 3 x 4 factorial design, made up of three treatments with four repeats per treatment. 200 first feeding fry were randomly allocated to each of the 12 tanks, with four tanks assigned as repeats to each treatment. The details of the respective treatments are presented in Table 3.7.

3.3 Experimental Material: Fish Stocks

3.3.1 Broodstock

The Mozambique tilapia (*Oreochromis mossambicus*) 150, used during the study was obtained from the general stock of the Division of Aquaculture at Wegevallen Experimental Farm, at Stellenbosch University. Breeding fish were conditioned to spawn by maintaining water temperature in the optimal range (28±2^{o}C). Broodfish was fed twice daily (Rana, 1988) on a standard commercial tilapia grow-out feed containing 25% crude protein (NUTROSCIENCE (PTY) LTD, Malmesbury, South Africa).

3.3.2 Experiment I

During the first week of October 2009 a total of 82 females (average weight 318g, average length 73mm) and 21 males (average weight 416, average length 98 mm) from the general stock at the Welgevallen Experimental Farm were randomly selected from the broodstock. The fish were stocked in 2500L breeding tanks at a ratio of 4♀:1♂. Fertilized eggs were removed from the mouth of brooding females at 10-14 day intervals and were artificially incubated and hatched.

The fry were reared in a raceway system to an average weight of 40g on the basal Tilapia diet (Table 3.2) fed at a recommended daily ration of 2% body weight per day. In September 2010, 350 sexually matured males and 350 sexually females of size ranging from 20 to 45g were selected and randomly allocated to Experiment Ia made up of 55 tanks (550 fish) and Experiment Ib made up of 15 tanks (150 fish).

Five males and five females were selected and randomly allocated to each tank (120L, see Figure3.1 above). The fish were allowed to acclimatize for 7 days, before the experimental diet was introduced.

3.3.3 Experiment II

The above mentioned spawning procedure was repeated during February 2011 to obtain fry with an average weight of 6g. In April 2011 fish of unknown gender (i.e. undeveloped urogenital papilla) were selected.
A total of 1,650 fingerlings were randomly allocated to the 55 tanks at the density of 30 fish per tank. The fish were allowed to acclimatize for 7 days on the basal Tilapia diet (Table 3.2) before the experimental diets were introduced, and fed for 60 days. Experiment II was conducted from April 2011 to June 2011

3.3.4 Experiment III

In May 2011 a total of sixteen females (average weight = 97g ± 7g) and four males (average weight = 123g ± 5g) were randomly selected from fish originationg out of Experiment I and placed in four breeding tanks at a ratio of 1♂:4♀. As soon as a female was seen to be carrying eggs, the other fish was removed from the tank and the female left by herself to incubate the eggs. At day 5 most eggs were hatched and the female started opening her mouth.

Fry of whose yolk sac was fully reabsorbed (i.e. swim up fry), were randomly collected by scooping them out at different angles in the tanks with a hand net. The average weight of the fry was determined by using an electronic scale (Electronic Balance, UWE, HGS-300, Capacity: 300 x 0.01g, Serial # P9440). The total length (i.e. from the tip of snout to tail end) of 10 fry were also measured using a measuring board.

In this trial, 200 fry were allocated per tank for each treatment, and each treatment was replicated 4 times (i.e. 12 tanks per treatment). A total of 2400 fry were thus allocated to Experiment IIIa and fed with 15g Pawpaw diet, but Experiment IIIb fry were fed 15g Moringa diet with same number of fish (2,400).

Successful sex change or reversal requires newly hatched fry (9-12mm total length) that are presumed to have sexually undifferentiated gonads. So, as soon as fry were observed to have hatched, the female parent was removed from the tank, leaving the fry behind. Most of these fry were yolk-sac free or had yolk attached to the body, without external feeding. The post hatch fry took a period of 9-12 days for consumption or depletion of the yolk, before commencement of exogenous or first feeding. Feeding with the experimental diets commenced a day after stocking was done. By this time the fry were 10-13 day old, with the yolk sac reabsorbed in most cases. The growth of tilapia fry depends on many factors, including stocking density, food and feeding regimes, photoperiods and water flow and replacement, and all these factors were provided within optimal range.

3.4 Experimental Material: Diets

3.4.1 Basal Diet

Each experimental diet consisted of a basal diet component and an experimental plant seed powder component. The basal diet (BD) used throughout the study was the commercial Tilapia Starter Feed (Aquanutro™), that was formulated and manufactured by Nutroscience (Pty) Ltd, Malmesbury, South Africa. The composition of the basal diet is presented in Table 3.2. The pelleted basal diet was ground into fine

powder, using a crasher (Drostsky S1, Mittal, SA), to which a predetermined quantity of the experimental compound was then added when the treatment feeds were prepared. The powdered basal diet was kept in a polystrene sac with a black plastic bag fitted inside and stored in the feed store of Feed Technology Unit, Division of Aquaculture, Stellenbosch.

Table 3.2 The nutritional composition of the Basal Diet (BD)

Diet components	Content (g/kg)
Protein	400 (minimum)
Moisture	120 (maximum)
Lipid	80 (minimum)
Fibre	40 (maximum)
Phosphorus	7 (minimum)
Calcium	30 (maximum)

3.3.2 Collection and Preparation of Experimental Compounds

The Plant Materials

Pawpaw (*Carica papaya* Linn. Caricaceae) and Moringa (*Moringa oleifera* Lam., Moringaceae) seeds were used as experimental compounds in the study.

Pawpaw (C. papaya)

Pawpaw seeds (Plate 3.2a) were obtained from whole fruits processed at the Fruit & Veg City Store in Stellenbosch, South Africa. The exact cultivar from which the seeds were collected, could not be ascertained due to the nature or conditions under which they were acquired. However, the most common cultivated Pawpaw in South Africa is 'Hortus Gold' cultivar, which was launched in the early 1950's (De La Cruz *et al.*, 2003).

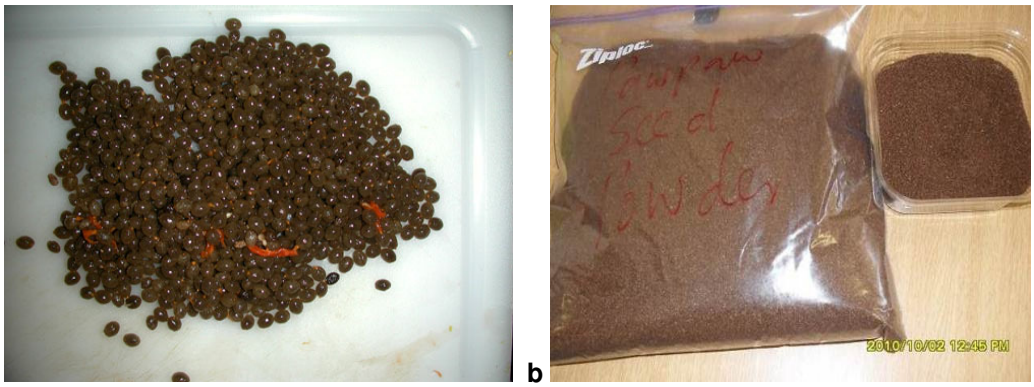

Plate 3.2 Pawpaw (a) fresh seeds and (b) dried powder, used to prepare the experimental diet

Fresh seeds were collected from the shop over a period of 3 months and blended into a paste using a laboratory blender (ATO MIX, Serial #703449, MSE Model, London). The paste was then oven-dried for 12 hours at 60 ℃ in an Electrocool oven (Envirowatch 5, CFE, South Africa), at a relative humidity of 15%. The fine powder form (Plate 3.2b) was obtained by sieving the dried powder with a sieve (Universal Laboratory Test Sieve, SABS ISO3310, Model Minor, Serial number 157-07), and loading the powder into air-tight double zipper plastic bags (Ziploc, SC Johnson & Son, SA (Pty) Ltd), which were then labelled and stored in a cool dry area until further use.

Moringa (M. oleifera)

Dry Moringa seeds were supplied by the Agribusiness in Sustainable Natural African Plant Products (ASNAPP) branch in Zambia. The method used to prepare the Moringa seed powder (Plate 3.3) was similar to the procedure used for Pawpaw.

Plate 3.3 Moringa (a) fresh dehusked seeds, (b) dried powder, used to prepare the experimental diet

3.3.3 Preparation of Experimental Diets

Experimental diets were prepared on a two weekly basis to ensure the availability of freshly made feeds throughout the study. Five kg of the basal diet and the predetermined quantity (i.e. inclusion levels 0.5, 1.0, 5.0, 10.0, & 15.0g) of the Pawpaw or Moringa seed powder was measured out, and both components were then thoroughly mixed for 10 minutes by using a feed mixer (Macadams Baking Systems, SM401). An amount of 800 ml of warm water at 60^{o}C was added to ensure that the mixture contained enough moisture for the extrution process. The pellets were produced using an Extruder with a 2mm die and oven-dried for 12 hours at 60^{o}C.

For the hormone treated feed a 17-α methyltestosterone stock solution was prepared by dissolving 60mg of 17MT in 600ml of 95% alcohol. The required amount of stock solution of 300mg was sprayed to the prepared pellets of 5kg and mixed thoroughly for the hormone to penetrate all particles. The moist feed was allowed to air-dried in the feed preparation laboratory of Feed Technology Unit (DA-WEF, Faculty of AgriScience), out of direct sunlight until dried.

The feed for first exogenous feeding fish (9 – 12 day post hatch) was prepared as a fine powder by grinding and sieving the basal diet pellets using the laboratory blender and a sieve (Universal Laboratory Test Sieve, SABS ISO3310, Model-MINOR, Serial #:1575-07).

The basal diet served as a negative control treatment. Positive treatment was prepared by adding set quantities (i.e. 30g/5kg) of 17MT to the basal diet powder. After drying, the experimental feeds were packed in air-tight plastic bags labelled and stored under dark, dry conditions until ready to use.

Experiment Ia: Pawpaw and Moringa treatments

The composition of the experimental diets for Experiment I, during which the effect of the crude seed powder on the gonadal activity of sexually mature Mozambique tilapia (*Oreochromis mossambicus*) was investigated, is presented in Table 3.3.

Table 3.3 Inclusion levels of Pawpaw (*C. papaya*) and Moringa (*M. oleifera*) seed powder in the experimental diets fed during Experiment Ia.

Treatments	**Designation**	**Inclusion level of experimental compound (g/kg of Basal Diet, BD)**				
i) Basal diet (BD)*	$C_{(I-V)}$	No dosage level for Control Diet				
ii) BD + Pawpaw	$PP_{(I-V)}$	P(0.5)	P(1.0)	P(5.0)	P(10.0)	P(15.0)
iii) BD + Moringa	$MO_{(I-V)}$	M(0.5)	M(1.0)	M(5.0)	M(10.0)	M(15.0)

*Basal Diet: Commercial Aquanutro Tilapia starter diet. Five repeats per treatment (I-V). Fish per tank = 10.

Experimental Ib: Pawpaw + Moringa + 17MT treatment

The composition of the experimental diets for Experiment Ib, to test for synergistic effects of P and M on the gonadal activity of sexually mature Mozambique tilapia, is presented in Table 3.4.

Table 3.4 Inclusion levels of Pawpaw (*C. papaya*) and Moringa (*M. oleifera*) seed powder in the experimental diets fed during Experiment Ib.

Treatments	**Designation**	**Inclusion level of experimental compounds (g/kg of basal diet, BD)**
i) Basal diet (BD)*	$C_{(I-V)}$	No dosage level for Control Diet
ii) BD + 17α-Methyltestosterone	$MT_{(I-V)}$	0.06g 17αMethyltestosterone
iii) BD + 10g Pawpaw + 5g Moringa	$P10M5_{(I-V)}$	10g Pawpaw + 5g Moringa Seed Powder
iv) BD + 5g Pawpaw + 10g Moringa	$P5M10_{(I-V)}$	5g Pawpaw + 10g Moringa Seed Powder

* Basal Diet: Commercial Aquanutro Tilapia starter diet. Five repeats per treatment (I-V). Fish per tank = 10.

Treatment 1 consisted of the BD (negative control), Treatment 2 consisted of 17-α methyltestosterone that was included at a concentration of 0.06g/kg BD (positive control), Treatment 3 consisted 10.0g P and 5.0g M added per kg BD, and Treatment 4 consisted of 5.0g P and 10.0g M per kg BD. The respective treatments are presented in Table 3.4.

Experimental II: Pawpaw and Moringa treatment

The composition of the experimental diets for Experiment II, during which the effect of the seed powder on the gonadal activity of sexually immature Mozambique tilapia (*Oreochromis mossambicus*) was investigated, is presented in Table 3.5

Table 3.5 Inclusion levels of Pawpaw (*C. papaya*) and Moringa (*M. oleifera*) seed powder in the experimental diets fed during Experiment II.

Treatment	**Designation**	**Inclusion level of experimental compounds (g/kg basal diet) and designation**				
(i) Basal diet (BD; Control)*	C $_{(I-V)}$	No dosage level for Control Diet				
(ii) BD + Pawpaw seed powder	P $_{(I-V)}$	P(0.5)	P(1.0)	P(2.0)	P(5.0)	P10.0)
(iii) BD + Moringa seed powder	M $_{(I-V)}$	M(0.5)	M(1.0)	M(2.0)	M(5.0)	M(10.0)

* Basal Diet: Commercial Aquanutro Tilapia starter diet. Five repeats per treatment (I-V). Fish per tank = 30.

The results from Experiment I indicated that an inclusion level of 5 to15g Pawpaw/Moringa seed powder per kg BD showed effect on gonadal integrity. Consequently, the inclusion levels of both the Pawpaw and Moringa seed powder were adjusted to include an inclusion level of 2.0 g P or M/kg, and the 15.0g P or M/kg level was omitted.

Experiment III: Pawpaw and Moringa treatments

The experimental diets for Experiment III are presented in Table 3.6. The diets were prepared for first exogenous feeding fish. A fine powder was prepared by crushing the prepared pelleted feed (as described above) using the laboratory blender and sieved.

Table 3.6 Inclusion levels of Pawpaw (*C. papaya*) and Moringa (*M. oleifera*) seed powder in the experimental diets fed during Experiment III.

Treatments	**Designation**	**Dosage (g/kg of basal diet, BD)**
(i) Basal diet (BD)*	NC $_{(I-IV)}$	No dosage level for Control Diet
(ii) BD + 17α-methyltestosterone	MT $_{(I-IV)}$	0.06g 17α-methyltestosterone
(iii) BD + Pawpaw	P15 $_{(I-IV)}$	15g Pawpaw
(iv) BD + Moringa	15M $_{(I-IV)}$	15g Moringa

*Basal Diet: Commercial Aquanutro Tilapia starter diet. Four repeats per treatment. Fish per tank = 200.

At the start of each trial 150g of experimental diet was weighed into a 500ml sampling bottle, labelled and covered with aluminium foil to prevent biochemical or photochemical reaction of the feed (personal communication from Mr Lourens deWet, Feed Technology Unit, Division of Aquaculture, Stellenbosch University).

3.5 Experimental Methods

3.5.1 Feeding

Experiment I and II

In Experiments I and II fish were acclimatized for 7 days, during which they were fed with a commercial tilapia diet once a day to sustain them. Feeding of the fish with the experimental diet commenced after the 7th day of stocking and lasted for 60 days. Fish were fed *ad libitum* (i.e. to satiation, Baker, 1984) in three instalments at 0800-0900h, 1200-1300h and 1600-1700h.

The health of the fish was managed by providing good water quality and ensuring that all the tanks were clean. The experimental unit was cleaned constantly throughout the duration of the study as explained in section 3.1. New water was added and disinfected with common salt every time the mechanical filter was cleaned.

Experiment III

In Experiment III fish were fed on the experimental diets as of a day after stocking, by which time they were 10 -13 days old and most of them had their yolk absorbed. Fish were fed ad libitum, 3-5 times a day for the 30 days duration of the trial, depending on the conditions in the tanks and feeding behaviour. After the 30th day feeding, the fish allowed to live on the basal diet only for 60 days to attain the size for external or manual sex determination.

3.5.2 Data recording

Experiment I

Data was recorded for external morphological traits, including total body length, weight and depth. Internal body organs (gonad and liver) were also weighed. The external body measurements were taken before stocking and at the end of the 60 days of feeding, whilst the internal organs were measured at the end of the trial only. Two fish (1male and 1 female) were randomly removed from each treatment batch (11 treatments 5 replicates).

The body weight of the fish was measured using an electronic weighing scale (Electronic Balance, UWE, HGS-1500, Capacity: 1500 x 0.05g, Serial #-T5912) to the nearest 0.01g. Both the total length (i.e. from the tip of snout to tail end) and depth (i.e. base of dorsal fin along the opercula line to the bottom of ventral section of the body) (Skelton, 2001), were measured to the nearest 0.01mm using a calibrated measuring board.

Internal body organs i.e. the testes and ovaries were weighed using electronic weighing scale (Electronic Balance, UWE, HGS-300, capacity: 300 x 0.01g, Serial # P9440) to the nearest 0.01g and fixed in Bouin's solution for histological study.

Experiment II

Experiment II was conducted with fish of unknown sex (undeveloped urogenital papilla) for 60 days. The weight was taken using electronic balance (UWE, HGS-300, Capacity: 300 x 0.01g, Serial # P9440). Total length and body depth of the fish were measured using the calibrated measuring board, before and after the trial.

Sex determination and histology

After the 60-day trial four fish were randomly removed from each tank, and sexed into males and females using external features (genital papilla, the anal and dorsal fins). Sex was confirmed through examination of the gonads obtained by dissection.

Internal body organs i.e. the testes and ovaries were weighed using electronic weighing scale (Electronic Balance, UWE, HGS-300, capacity: 300 x 0.01g, Serial # P9440), to the nearest 0.01g and fixed in Bouin's solution for histological study

Growth

Growth was monitored only in Experiment II. After the 60-day trial, 5 fish from each of the 55 tanks were selected and pooled according to the various experimental Pawpaw and Moringa diets (0; 0.5; 1.0; 1.5; 2.0; 5.0; and 10.0g/basal diet). They were reared and observed for biological, reproductive and social behaviour for another 60 days. A standard commercial tilapia grow-out feed containing 25% crude protein was fed *ad libitum* thrice daily to the fish for 60 days (July to September 2011). The total length, body depth and body weight were taken before they were pooled. After the 60 days, total length, body depth and body weight were taken again.

Experiment III

In experiment III, after the 30 trial, fish were fed on basal diet for another 60 days. At the end of the 90 days (i.e. 30 days on experimental diet and 60 days on basal diet diet), 50 fish were randomly sampled from each aquarium and their sex determined. At the end of the 90 day experimental period (i.e. 30 days treatment and 60 days control diet) gender could be comfortably determined in the fish.

Gender was determined by the presence/absence of specific external morphological characteristics, i.e. genital papillae, and the length and shape of the dorsal and anal fins. Gender was confirmed by gonadal examination, as described in 3.6.1 below. The total number of males and females identified after dissection were recorded. Also 4 fish (2males and 2 females) were randomly taken from each group of 50 fish, and the total length, body depth and body weight measured, and their gonads fixed in Bouin's solution.

Mortality was monitored and recorded for all the experiments and the data was used to calculate survival rates. Dead fish were removed from the tank on daily basis as a standard management procedure.

3.5.3 Observational Studies

Fish Behaviour

This study was conducted on experiments I and II, and looked at the social interaction such as black body formation, emergence of dominant males and territorial establishment. In Experiment I, the fish were sexually matured, trial period (i.e. October to December is summer in Southern Hemisphere), which is the breeding season for the fish in wild (Bruton and Bolt, 1975); thus this behavioural study was conducted. For Experiment II 30 fish were stocked in the aquaria at a smaller size but high density than Experiment I (n=10). During stocking, available fish weighing 5-10g were old, so after 3days in glass tanks some started breeding. After been able to stock the tanks with fish (2-8g), this study then looked at dark male formation and establishment of territories in the tanks, for the 60-day period.

3.6 Histological Methods

3.6.1 Specimen Collection

Histological study was conducted in experiments I and II for testes, ovaries and livers examination. After taking body measurements of the fish, they were anaesthitized with clove oil and then dissected. Each specimen was dissected with the aid of a pair of small scissors inserted through the vent. Also a semi-circular cut was made laterally on the side of the specimens for better observation. In each fish the state of the gonad (testes and ovaries) was visually observed for any deformity. For instance, in an ovary with matured eggs, the colour, relative size and shape of both gonads, were considered. In a testis, for instance, the structure, shape and texture were examined for any constriction. Any form of deformity or unusual appearance (e.g. sizes of gonads) was noted and photographed.

The testes and ovaries were then weighed and data recorded, and fixed in Bouin's solution for preservation. In experiment II the liver was examined for colour change and any form of physical damage in both sexes, after which it was weighed, and in Bouin's solution for histological study investigation or examination.

3.6.2 Preparation, sectioning and staining

The specimens preserved in Bouins solution were taken to the Division of Anatomy and Histology, Department of Biomedical Sciences, Tygerberg campus, Stellenbosch University, near Cape Town about 40-45minutes drive (by car) from the main university campus at Stellenbosch for histological analysis.

All the tissues in Bouin's were processed in an automatic tissue processor (SHANDON ELLIOT, Duplex Processor) by immersion in a serial dilution of alcohol (i.e. 80%, 90% and 100%), chloroform and molten paraffin wax, for 17 hours in the laboratory. The tissues were then embedded in paraffin wax at 60 ^{o}C, in an embedding instrument (LEICA EG 1160).

Using a microtome (Model, Bright 5040), 5µm -8µm thickness sections were cut. All tissues were oriented on the wax block in such a way to produce transverse sections (TS). However, few tissues of the testis were cut in longitudinal sections due to the size of the organ. For each specimen at least 3 sections were placed on a glass slide in warm water. All the slides were stained for 25 minutes using LEICA AUTO STAINER, XL, with Ehrlich's haematoxylin and counterstained with eosin. Cover slips were attached to the slides with DPX mounting solution. DPX mountant for histology is a mixture of distyrene, plasticizer, and xylene (see Appendix I for details processing solutions, steps, and staining procedure).

3.6.3 Slide examination

The slides were viewed or examined using a light compound microscope (OLYMPUS CH-30). Using a computer programme NIS Elements BR 3.0 (Driver Nikon Ds-U1, version 5.547) the image was enlarged onto a monitor for analysis. Photomicrographs of images shown on the screen were taken with Nikkon Digital Sight, DS Fi1 camera mounted on Zeiss-OLYMPUS CH-30 microscope. On the NIS Elements BR 3.0 programme, the images were resized, sharpened and colour adjusted prior to photographing. The images were combined into plates labelled on Microsoft Office Power Point, 2010, using a free hand.

3.6.4 Visual defects of gonads (Experiment I & II)

The diagnosis of any effects or defects described for the treatment groups was done in comparison with controls. All the slides prepared from treatments were examined under the microscope (see 3.6.3). The microphotographs taken were compared with those of Coward and Bromage (2000), Msiska (2002), Ekanem and Okoronkwo (2003), Morrison *et al.* (2006), Jegede and Fagbenro (2008) and Abdelhamid *et al.* (2010) described and labeled. The details of microphotographs and description of the visual defects are presented in Chapters 4 and 5.

3.6.5 Histological Gonadal Damage Grading

OECD (2009) explained that histopathology is a descriptive science, and therefore somewhat subjective. However, histopathological evaluations of the same study by any qualified pathologist should identify the same treatment-related findings (Crissman *et al.*, 2004). Therefore, a diagnostic criteria for histopathological evaluations or analysis of the study that was encountered has been defined.

These criteria are based on certain consistent histopathological changes that occur in tilapia gonads in response to chemical exposure. The criteria are graded for severity on a numerical scale (Tables 3.7 and 3.8), as seen under the microscope in comparison with Morrison *et al.* (2006) and the control of this a study, using OECD (2009), as a guide for the description.

Table 3.7 Grading criteria for the evaluation of the influence of phytogenic feed additives on gonadal development in female Mozambique tilapia (*Oreochromis mossambicus)*

Grading class (score assigned)	**Condition**	**Description of characteristic evaluated (assessed)**
0 to 1	Normal, no effect	i) Normal ovary tissue containing all stages of oocyte development ii) Lumen in center of ovary filled with mature oocytes iii) Thick intact ovarian wall iv) No pathological lesions observed v) Gonad arteries, veins and nerves visible at high magnification,
1 to 2	Minimal effect	i) Normal ovary containing developing oocytes ii) Ovum lamellae and follicular lining intact iii) Less visible atretic follicles iv) Increase vacuolated oocytes v) Decrease in the number and stages of developing oocytes. vi) Decrease in number of developing oocytes with a decrease in yolk particle content
2 to 3	Medium	i) Slight liquefaction of cytoplasm of oocytes ii) Depletion of ripe oocytes iii) Increasing degeneration in wall of oocytes iv) Nucleus losing its circularity v) Abnormal shape of oocyte in vitellogenic stage vi) Coagulation necrosis in yolk granules vii) Increasing atretic follicles
3 to 4	High	i) Depleted yolk particles ii) Atresia of oocytes iii) Migratory nucleus in oocytes loses its circularity iv) Lysis of oocytes v) Pronounced coagulative necrosis vi) Severe liquefaction of yolk, with large vacuoles of ripe oocytes vii) Severe irregular wall of oocytes

Table 3.8 Grading criteria for the evaluation of the influence of phytogenic feed additives on gonadal development in male Mozambique tilapia (*Oreochromis mossambicus)*

Grading class (score assigned)	**Condition**	**Description of characteristic evaluated (assessed)**
0 to 1	Normal, no effect	i) Normal gonad showing standard testicular tissue with normal sperm cell distribution ii) Thick intact testicular wall iii) Normal seminiferous tubules with visible normal spermatocytes and spermatids. iv) Presence of intact collecting duct and fibrous connective tissue. v) Gonad arteries, veins and nerves visible at high magnification
1 to 2	Minimal effect	i) Normal maturing sperm cells. ii) Seminiferous tubules not entire (intact). iii) Decreasing number of spermatogonia and spermatids. iv) Minimal Increase in interstitial cell component of testis. v) Onset of degeneration in testicular tissue
2 to 3	Moderate effect	i) Disintegrating sperm cells ii) Increasing degeneration of seminiferous tubules iii) Collecting ducts devoid of spermatids iv) Increasing interstitial cells v) Increasing necrosis of testicular cells vi) Swollen sperm cells nuclei
3 or 4	Pronounced effect	i) Atrophied seminiferous tubules ii) Focal necrosis in sperm cells iii) Hemolysis in testes tissues iv) Greater increase in interstitial cells v) Degenerating seminiferous tubules into the testicular tissues vi) Collapsed collecting duct vii) Seminiferous tubules free of spermtocytes and spermatids viii) Severe disintegration of sperm cells

The respective preparations were scored according to the following guidelines:

For assigning a score of between 0 and 1, more than three traits have to be observed.

For assigning a score of between 1 and 2, more than 3 of traits (ii) to (vi) had to be observed.

To assign a score of between 2 and 3, more than 4 of the criteria had to be observed.

For assigning a score of between 3 and 4, more than 5 of the traits had to be observed.

The criteria were used to place the slides in their respective state, and the frequency of each grade was tallied, recorded, and statistically analysed.

3.7 Statistical Analysis

3.7.1 Body Measurements

Data on body measurements (the total length, body depth and body weight) for all experiments (I, II & III) were analysed by calculating means and standard deviations (Bhujel, 2008), Scientific Calculator, (SHARP EL-W53H)

Survival in the 3 experiments was determined from the relation

$$\text{Percent survival} = \frac{\text{Final Number of Fish}}{\text{Initial Number of Fish}} \times 100$$

(Source: Huang and Chin, 1997, modified)

For experiment III a Chi-squared analysis was used to test for significant deviation from the expected 1 male: 1 female sex ratio (Bhujel, 2008) at a 5% probability level for each replicate group as well as for the pooled data for each treatment.

3.7.2 Diets

A one-way analysis of variance (ANOVA) was performed to test for the significance of variance for the body weight, length and depth traitsof the fish between different treatments using statistical programme (SAS Version 9.1).

Correlation between condition of the gonads and feeding of the various dosage levels of the plant additive was also estimated. Regression analysis was also determined on the response of various dosage levels to the growth in experiment II.

Correlation between gonadal effect/activity and feeding of the various dosage levels of the plant additive was also estimated. Regression analysis was also determined on the gonadal response to various dosage levels. Fish weight, gonad weight and effect of the various plant additives was also compared using one-way ANOVA (using SAS Version 9.1).

The statistical results generated from the SAS programme are shown in Appendix 4.

3.7.3 Histology

Analysis of the of internal organ measurements (the gonad and liver weights) of all fish in experiments I and II taken for histological studies were done by calculating means and standard deviations. Gonadosomatic index for all these fishes were also calculated using the relationships below:

$$\text{Gonadosomatic Index (GSI)} = \frac{\text{Gonad weight (wet) (g)}}{\text{Body weight (wet) (g)}} \times 100$$

(Source: Coward and Bromage, 1999; Celik and Altun, 2009)

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Chapter 4

The Effect of Pawpaw Seed Meal and Moringa Seed Meal on the Gonadal Development of Sexually Mature Mozambique Tilapia (*Oreochromis mossambicus*)

Abstract

In Sub-Sahara African (SSA) countries, culture efforts of tilapia are complicated by restricted access to appropriate methods to control the precocious nature of tilapia reproduction, and the impact thereof on the cost-efficient management of such systems. Uncontrolled breeding in tilapia results in overstocking of culture systems, which result in stunted growth and a variation in market size. Alternative methods that will provide suitable and cost-effective options to resource-poor communities in SSA to culture tilapia cost-efficiently were investigated. Pawpaw (*Carica papaya*) and Moringa (*Moringa oleifera*) seeds are reported to have an antifertility effect in certain livestock species. The study investigated the potential of Pawpaw (*Carica* papaya) seed meal (P) and Moringa (*Moringa oleifera*) seed meal (M) as potential endocrine disrupters to be used as an antifertility treatment in Mozambique tilapia (*Oreochromis mossambicus)*. Pawpaw and Moringa seed powder were included at 0, 0.5, 1.0, 5.0, 10.0 and 15.0 g/kg of a commercially formulated tilapia feed. The experimental diets were fed to sexually mature male and female Mozambique tilapia, weighing 20-45g, for a period of 60 days to evaluate the effects of P and M on gonadal function and integrity. Gonad integrity was not affected in fish that received the control diet (0g P/kg), and no gonadal lesions were observed. Diets containing 5.0, 10.0 and 15.0g /kg, of P and M respectively, influenced gonad integrity considerably, resulting in atrophy of the seminiferous tubules, as well as disintegration of sperm cells and necrosis of testicular cells. In the female fish atresia of oocytes were observed – at all inclusion levels. Inclusion levels of P and M showed an ovotestis a a testicular tissue.The study indicated that P and M at inclusion levels of 5.0/kg basal diet and higher have a significant antifertility effect, affecting gonad integrity and gamete production in sexually mature *O mossambicus*, suggesting that P and M can be used as potential sterility-inducing agents to control precocious breeding and thus overstocking in tilapia culture systems. Sustainable availability of Pawpaw and Moringa seeds in SSA countries, contributes to the attractiveness of this product as a potential antifertility treatment for tilapia.

4.1 Introduction

Aquaculture is the fastest growing food-producing sector which is considered as having the greatest potential to meet the growing demand for aquatic food, which by estimation would provide more than 50 percent of global food fish consumption by the end of 2012 (FAO, 2011; 2012). Available FAO data indicates that in 2010, world aquaculture production was 60 million tonnes (i.e. excluding aquatic plants and non-food products), which is considered as an all-time high with an estimated total value of US$119 billion (ZAR 1,059.1 billion). In 2011, production increased to 154 million tonnes, of which 131 million tonnes was used as food. The FAO has estimated that, globally, fish provides about 3.0 billion people with almost 20 percent of their animal protein, and 4.3 billion people with about 15 percent of such protein. Fisheries and aquaculture provided livelihoods and income for an estimated 54.8 million people engaged in the primary sector of fish

production in 2010, of which an estimated 7 million were occasional fishers and fish farmers (FAO, 2011, 2012).

Current figures show that aquacultural species is dominated by two freshwater fish groups i.e. the carps and tilapias, producing 24, 237, 303 metric tonnes and 3, 497, 391 metric tonnes respectively (FAO, 2012). Tilapia is described as the most prominent aquaculture species of the 21st century (Shelton, 2002; Shelton and Popma, 2006), and has become one of the most important food fishes of the world. Tilapia is the second most important farmed fish globally, following carp, and is produced and consumed in all the continents and more countries than most other species, serving as a major protein source in many of the developing countries (Gupta and Acosta, 2004). The tilapia is native to Africa and Middle East; however, due to introduction they are now being farmed commercially in almost 100 countries worldwide, with about 98% of tilapia production outside their original habitats.

It has been reported that since the 1990s the tilapia industry has grown to gain international prominence, being served in expensive restaurants and grocery stores in industrialised countries such as United States and European Union (Fitzsimmons, 2006). According to Norman-López and Bjørndal (2009), tilapia has made the market more heterogeneous than the other successful aquaculture species such as salmon and shrimp. Tilapia consumption in the USA has increased since 2000, becoming the second most popular fish in US retail stores, behind salmon, and the 5th most popular fishery product overall (Josupeit, 2010). From 2005 the USA has become the highest importer of tilapia (i.e. in whole frozen, fillet frozen and fillet fresh form); and in 2010, importation of tilapia products was estimated to reach a value of US$760,000,000 (ZAR 6.764 trillion) (Fitzsimmons, 2010).

Tilapia has unique characteristics that facilitate their cultivation in a variety of production systems, e.g. ponds, cages, raceways, and recirculation systems. The genus *Oreochromis* has culture advantages such as fast growth, adaptability to a wide range of environmental conditions and stresses, readily acceptance of artificial feed, as well as ease of production (Teichert-Coddington *et al.,* 1997; Gupta and Acosta, 2004; El-Sayed, 2006). According to Fitzsimmons (2010), tilapia culture is globally crossing cultural barriers due to the fact that the species can be grown in all types of water bodies, i.e. fresh water, brackish water as well as in marine water, requiring minimal fish meal. The dramatic increase in tilapia culture can be ascribed to the use of two tilapia species, Nile tilapia (*Oreochromis niloticus*) and Blue or Israeli tilapia (*O. aureus*), and various hybrid combinations of these species with the Mozambique tilapia (*O. mossambicus*) (Gupta and Acosta, 2004).

In a proper culture set up, most tilapia are capable of reproducing at an age of 5 to 6 months, and can spawn every 6 to 8 weeks at water temperatures between 25°C and 32°C. Unlike most aquaculture species such as catfish in which spawning is one of the major culture problems, spawning tilapias require minimum skill, technique and experience (Lim, 1989). Thus, Tilapia are able to breed freely in captivity. The major problem of tilapia culture however, is, the tendency of females maturing and reproducing at a very small size. Under fast growing conditions in culture ponds, *O. mossambicus* may reach sexual maturity in as little as three

months of age, weighing as little as 60 to 100 g. Sexual maturity can be delayed by a month or two when growth is slow in farm ponds, but it was observed that fish may spawn at weights as low as 20 g. However, in poorly fertilized ponds sexually mature fish may be as small as 15 g (Mair and Little, 1991; Popma and Lovshin, 1996).

Rearing both sexes of tilapia in the same pond results in overpopulation, and the high prolific nature of tilapia results in an increased degree of competition between offspring and adult fish for food. This results in stunted growth, with the original stock yielding only a small percentage of marketable fish weighing less than 100grams (Mair and Little, 1991; Toguyeni *et al.*, 1997).

It is well established that sexual maturity in tilapia is a function of age, size and environmental conditions. However, early sexual maturation in tilapia poses a potential problem for culture, for available energy is diverted from somatic growth to gonad development and reproductive activities such as territorial/courtship behaviour and gamete production (Popma and Lovshin, 1996).The effect is that the production of large table-size fish becomes illusive in mixed culture practices, with recruits that may constitute up to 70% of the total harvest weight (Rakocy and McGinty, 1989; Mair and Little, 1991). Of the 3 dominating *Oreochromis* species, the Mozambique tilapia attain sexual maturity at a smaller size and younger age than the Nile and Blue tilapia, with stunting observed in Mozambique tilapia (Pullin, 1988). Stunting and inability to obtain marketable size in tilapias is a big problem in their cultivation, and this contributes to the absence and low popularity of their culture in Sub-Saharan Africa (SSA). Studies over the last two decades in SSA have indicated that monosex tilapia production systems appear to be more financially viable in commercial small-scale culture (Standtler *et al.,* 2008), however, tilapia polyculture with catfish is the preferred system in rural small-scale culture in countries such as Ghana.

The early maturation and precocious breeding nature of tilapia in culture systems necessitates the development of techniques to control unwanted reproduction (Phelps, 2006). Mair and Little (1991) listed the existing methods for the control of tilapia reproduction as intermittent harvest by removing parent stock; manual sexing before sexual maturation; stocking with predatory/piscivorous fish in grow-out ponds; stocking of fish at high densities; stocking of tilapia in net cages with a mesh size larger than 2.5 cm; growing in salinity higher than 25ppt for some tilapia species; sterilization through application of heat, cold or pressure shocks to fertilized eggs, and lastly hybridization of *Oreochromis* species.

Several studies have indicated that both natural and man-made chemicals that enter aquatic water are toxic to aquatic life (Pavlov *et al.,* 2009). Substances that occur in agricultural by-products, industrial waste and untreated sewages that drain into streams, rivers, lakes and other water bodies through precipitation and dry deposition, stormwater transport of fertilizers, and road silt and direct disposal (Allan, 1995), have the ability to interfere with endocrine systems and hormonal activities of all animals including man and fish. These chemicals are referred to as endocrine disrupting chemicals (EDC). In the aquatic environment, EDCs are easily bioavailable to fish through a variety of exposure routes, including aquatic respiration, osmoregulation, maternal transfer of contaminants in lipid reserves of eggs or yolk, dermal contact with contaminated

sediments and the ingestion of contaminated food (Jobling *et al*., 1998; Mills and Chichester, 2005; Vajda *et al*., 2011).

Previous studies have established that fish populations routinely exposed to EDCs *in situ* experience changes in population structure, with these changes evident at the larval or developmental stages, disrupting sexual development, behaviour and fertility (Mills and Chichester, 2005; Vajda *et al*., 2005). Continuous exposure to EDCs leads to alteration of their reproductive physiology and morphology which include changes in or skewed sex ratios, incidence of reproductive defects in larvae, egg-producing cells in the male testis (ovo-testis), reduced gonadal (i.e. testis and ovary) growth rates and size, reduced female reproductive tracts, feminisation of reproductive ducts, reduced gamete quality, degeneration of testicular germinal cells, increased liver size, increased levels of and vitellogenin (egg-yolk protein) in male blood plasma (Jobling *et al*., 1998; Mills and Chichester, 2005; Nash *et al*., 2006; Vajda *et al*., 2011).

Endocrine disruption also can be caused by naturally occurring chemicals or products (human and animal waste, e.g. estrogen), which could be steroidal or non-steroidal (Sassi-Messai *et al*., 2009; Vadja *et al*., 2011). Medicinal supplements, such as those recommended for estrogen replacement therapy in post-menopausal women, contain high levels of phytoestrogens. Industrial activities such as pulp and paper production also can release large quantities of phytoestrogens which have been found to affect reproductive system and function in fish. The degradation of vegetable matter and paper products in wastewater treatment plants also may contribute to releases of phytoestrogens into the aquatic ecosystem (Takurah and Takemura, 2011). One major by-product that comes out of pulp and paper mill is β-Sitosterol (a major plant sterol), which is linked to induced production and increased levels of vitellogenin in fishes (Pait and Nelson, 2002).

According to Moutsautsou (2007) and Patisaul and Jefferson (2010), phytoestrogens are a large family of plant-derived estrogens possessing significant estrogen agonist/antagonist activity. Generally, phytoestrogens are naturally occurring molecules encompassing several classes of compounds, i.e. the flavonoids (flavonols, isoflavonoids, and isoflavans), coumestans (coumestrol), lignans, saponins, and triterpenes (Patisaul and Jefferson (2010) The most common isoflavonoids are genistein and diadzein,and the flavonoids are quercetin and kaempherol, which are widely distributed in the plant kingdom (Mazur, 2000). Phytoestrogens are found in a variety of plants, but are most abundant in leguminous plants. Legumes are present in almost every diet throughout the world, and in addition to the seeds many other parts of the plant are also edible (Kurzer and Xu, 1997; Mazur *et al*., 1998). From among the diversity of 'plant estrogens', the flavonoids and coumestans have been identified as the most common estrogenic compounds in these plants (Price and Fenwick 1985; Mazur, 2000).

Pawpaw (*Carica papaya* L.), and Moringa (*Moringa oleifera,* Lam),are economically important food plants of the tropics and subtropics, highly valued in terms of industrial, medicinal, pharmacological and nutritional use. Several studies and reports show that all parts of these plants and their extracts possess rich sources of phytochemicals including ascorbic acid, flavonoids, phenolics and carotenoids, oestrogenic substances such

as β-sitosterol, iron, calcium, phosphorus, copper, vitamins A, B and C, α-tocopherol, riboflavin, nicotinic acid, folic acid, pyridoxine, β-carotene, protein, and in particular essential amino acids such as methionine, cystine, tryptophan and lysine, a number of vitamins and minerals and numerous phytochemicals, which contribute significantly to their high medicinal and pharmacological activities (i.e. antioxidant, therapeutic), and nutritional values (Anwar *et al*., 2007; Krishna *et al*., 2008; Kumar *et al.,* 2010).

The reported phytoestrogens identified in Pawpaw plant parts include β-sitosterol, saponins, and flavonoids (Krishna *et al*., 2008) and that of Moringa plant parts include saponins, triterpenes, e.g. oleanolic acid-3-glucoside and flavonoids (quercetin and kaempferol) (Anwar *et al*, 2007; Kumar *et al.,* 2010). In a review by Kumar *et al*., (2012), the authors indicated that oleanolic acid-3-glucoside and β-sitosterol are some of the phytochemicals that have shown 100% antifertility activities, which are also present in Moringa plants. Ekanem and Okoronkwo (2003), Jegede and Fagbenro (2008) and Abbas and Abbas (2011) all mentioned that pawpaw seeds contain active ingredients such as caricacin, an enzyme carpasemine, a plant growth inhibitor, and oleanolic glycoside. These authors adduced that, Das (1980) contended that oleanolic glycoside is an active component of Pawpaw seed, which had been found to cause sterility in male rats. According Fransworth *et al*. (1975), the active principle responsible for antiimplantation effect of papaya seed in female rats, might be 5-hydroxytryptamine. It has been established that Pawpaw contains these phytochemicals β-sitosterol, saponins, and flavonoids (Krishna *et al*., 2008), which are considered as phytoestrogens.

Soejarto *et al*. (1978) categorized Moringa as an antifertility plant. In a review by Anwar *et al.* (2007), the parts of the plant listed as having or showing abortifacent or antifertility property include root, gum and flowers, excluding stem bark, leaves and seeds. However, some earlier studies on atifertility profile of Moringa species had included leaves (Nath *et al*., 1992) and stem bark (Skukla *et al*., 1988; 1989). Sethi *et al.* (1988) have noted that contraceptive potency of many plants varied considerably with respect to season, temperature and place of collection. Phytochemicals identified in Moringa include saponins, triterpenes, e.g. oleanolic acid-3-glucoside and flavonoids (quercetin and kaempferol) (Anwar *et al*., 2007; Kumar *et al.,* 2010), and β-sitosterol (Kumar *et al*., 2012).

The available information on attempts of using phytochemicals to prevent tilapia precocious breeding in production systems are at the experimental stages (Francis *et al*., 2002), using pure or refined forms. So more research needs to be conducted to know their potency and application in tilapia culture. Also, crude forms needs to be looked into, considering the inaccessibility of refined products in Sub Saharan Africa (SSA) countries.

The study therefore investigated the potential of crude Pawpaw and Moringa seed powder to be used as potential EDC's to control the precocious breeding of tilapia in culture systems, potentially enabling the use of this species in SSA for food security purposes. The choice of Pawpaw and Moringa was based on the fact that it is relatively abundant in tropical Sub-Saharan African (SSA) countries for example Ghana, easily accessible all year round, and using the seeds could also preserve the plant for environmental sustainability.

Moreso Pawpaw and Moringa as natural plants, are currently used in several traditional medicines (Raji *et al*., 2005; Anwar *et al.,* 2007; Bose, 2007; Krishna *et al*., 2008), with some level of safer utilization and handling issues.

The aims of the study were therefore to test the hypothesis that feeding fish with diets containing natural plant-derived estrogenic/androgenic compounds (i.e. Phytoestrogen) will affect the gonadal activity of tilapia in culture systems. The study investigated the effect of different inclusion levels of Pawpaw and Moringa seed meal on gonadal integrity and function in sexually mature Mozambique tilapia, ranging from 20-45g in size.

4.2. Material and Methods

4.2.1 Experimental Unit and Location

The experimental unit was a recirculating aquaria system (RAS) consisting of 72-glass tanks (see Chapter 3 for design and operation). It was built inside a greenhouse, which is part of the Division of Aquaculture facilities at Welgevallen Experimental Farm, Faculty of AgriSciences (FAS), located at 33°56' 33.95" S and 18°51'56.15 "E.

4.2.2 Experimental Layout

Experiment I.a: (Pawpaw and Moringa: 0, 0.5, 1.0, 5.0, 10.0 and 15.0 g/kg basal diet, BD)

Five test diets each of Pawpaw and Moringa were prepared by adding 0.5, 1.0, 5.0, 10.0 and 15.0 g of Pawpaw and Moringa seed powder to 1 kg of the powdered basal diet (Table 3.2) to obtain the Pawpaw Seed meal (P) and Moringa Seed meal (M). Thus 11 experimental diets as shown in Table 4.1, were prepared (5 each from P & M and 1 control). Experiment I was conducted between October and December 2010. The experiment was conducted using 550 sexually matured *Oreochromis mossambicus*, (20 - 45g; 275 males and 275 females), which were randomly distributed into 55 tanks, each receiving 5 males and 5 females. All the 11 treatments were replicated five times, representing 11 x 5 factorial experimental design.

Table 4.1 The experimental design (11x5=55 factorial) and feed preparation layout for evaluation of Pawpaw and Moringa Seed meal on gonadal activity of sexually matured tilapia (20-45g, *Oreochromis mossambicus*).

Treatments	Designation	Inclusion level of experimental compound (g/kg of Basal Diet, BD)				
i) Basal diet (BD)*	$C_{(I-V)}$	No dosage				
ii) BD + Pawpaw	$PP_{(I-V)}$	P(0.5)	P(1.0)	P(5.0)	P(10.0)	P(15.0)
iii) BD + Moringa	$MO_{(I-V)}$	M(0.5)	M(1.0)	M(5.0)	M(10.0)	M(15.0)

*Basal Diet: Commercial Aquanutro Tilapia starter diet. Five repeats per treatment (I-V). Fish per tank = 10.

Experiment I.b: (Interactive study: 0, 17MT, P10M5 and P5M10)

Three test diets and a control (Table 4.2) were used in this study. The first diet was a mixture of 10.0 g Pawpaw seed powder and 5.0g Moringa seed powder and 1 kg of the basal diet (P10M5 diet). The second was a mixture of 5.0 g Pawpaw, 10.0g Moringa and 1kg basal diet (P5M10). The third was a hormone (17-α methyltestosterone) incorporated into the basal diet. The experiment was conducted using 150 fish (20-45g; 75 males and 75 females), which were randomly distributed into 15 tanks, with each tank receiving 5 males and 5 females. This control was the basal diet in Experiment I. The 3 treatments and the control were replicated five times. The experimental design was a 4 x 5 factorial.

Table 4.2 The experimental design (3x5=15 factorial) and feed preparation layout for comparative evaluation of 17α- methyltestosterone and a mixture of Pawpaw and Moringa seed meal on (20-45g, *Oreochromis mossambicus*).

Treatments	Designation	Inclusion level of experimental compounds (g/kg of basal diet, BD)
i) Basal diet (BD)* only	C $_{(I-V)}$	No dosage
ii) BD + 17α-Methyltestosterone	MT $_{(I-V)}$	60mg 17αMethyltestosterone
iii) BD + 10g Pawpaw + 5g Moringa	P10M5 $_{(I-V)}$	10g Pawpaw + 5g Moringa Seed Powder
iv) BD + 5g Pawpaw + 10g Moringa	P5M10 $_{(I-V)}$	5g Pawpaw + 10g Moringa Seed Powder

* Basal Diet: Commercial Aquanutro Tilapia starter diet. Five repeats per treatment (I-V). Fish per tank = 10.

4.2.3 Experimental Materials

Fish

Mozambique tilapia (*Oreochromis mossambicus*) samples were obtained from the general stock resource at the Division of Aquaculture, Wegevallen Experimental Farm, Stellenbosch University. Seven hundred sexually matured males (350) and females (350), ranging in weight from 20-45g, which sexually matured were selected and stocked between the last week of September 2010 and the second week of October 2010 in 70 glass tanks, at a stocking density of 10 fish per tank (i.e. 5 males and 5 females). Two experiments were conducted simultaneously; in Experiment I.a 550 fish (275 males and 275 females) were randomly distributed into 55 tanks; and in Experiment I.b 150 fish (75 males and 75 females) were also randomly distributed into 15 tanks. They were allowed to acclimatize for 7 days, before the experimental diets are administered.

Plants

Pawpaw (*Carica papaya*) and Moringa (*Moringa oleifera*) (See Chapter 2: Figure 2.13 and Figure 2.14) seeds were used. Pawpaw seeds were obtained gratis from Fruit & Veg City Store, Stellenbosch, South Africa. Moringa seeds were purchased from Agribusiness in Sustainable Natural African Plant Products (ASNAPP), Zambia branch (i.e. ASNAPP-Zambia), which authenticated them.

Feeding

The fish were acclimatized for 7 days, during which time they were fed with a commercial tilapia diet once a day to sustain them. Feeding of the fish with the experimental diet commenced after the 7th day of stocking, and lasted for 60 days. The fish were fed *ad libitum* (i.e. to satiation) (Baker, 1984) in three installments at 0800-0900 h, 1200-1300h and 1600-1700 h.

The health of the fish was managed by providing good water quality and ensuring that all the tanks were clean. The experimental unit was cleaned constantly throughout the duration of the study as explained in section 3.1. New water was added and disinfected with common salt every time the mechanical filter was cleaned.

4.2.4 Histological Evaluation

Detailed protocol followed for the collection, preparation and processing, and evaluation of the histological slides prepared from the tissue samples obtained from fish sacrificed from the respective treatment groups has been described in Chapter 3.

4.2.5 Data recorded

The external body measurements were taken before stocking and at the end of the 60 days of feeding, whilst the internal organs were measured at the end of the trial only.

The body weight of the fish was measured using an electronic weighing scale (Electronic Balance, UWE, HGS-1500, Capacity: 1500 x 0.05g, Serial #-T5912) to the nearest 0.01g. Both the total length (i.e. from the tip of snout to tail end) and depth (i.e. base of dorsal fin along the opercula line to the bottom of ventral section of the body) (Skelton, 2001), were measured to the nearest 0.01mm using a calibrated measuring board.

Two fish (1male and 1 female) were randomly removed from each treatment batch (11 treatments 5 replicates). After taking body measurements of the fish, they were anaesthitized with clove oil and then dissected. Each specimen was dissected with the aid of a pair of small scissors inserted through the vent. Also a semi-circular cut was made laterally on the side of the specimens for better observation. Internal body organs i.e. the testes and ovaries were weighed using electronic weighing scale (Electronic Balance, UWE, HGS-300, capacity: 300 x 0.01g, Serial # P9440) to the nearest 0.01g and fixed in Bouin's solution for histological study.

The specimens preserved in Bouins solution were taken to the Division of Anatomy and Histology, Department of Biomedical Sciences, Tygerberg campus, Stellenbosch University, near Cape Town about 40-45minutes drive (by car) from the main university campus at Stellenbosch for histological analysis.

All the tissues in Bouin's were processed in an automatic tissue processor (SHANDON ELLIOT, Duplex Processor) by immersion in a serial dilution of alcohol (i.e. 80%, 90% and 100%), chloroform and molten paraffin wax, for 17 hours in the laboratory. The tissues were then embedded in paraffin wax at 60 ^{o}C, in an embedding instrument (LEICA EG 1160).

Using a microtome (Model, Bright 5040), 5µm -8µm thickness sections were cut. All tissues were oriented on the wax block in such a way to produce transverse sections (TS). However, few tissues of the testis were cut in longitudinal sections due to the size of the organ. For each specimen at least 3 sections were placed on a glass slide in warm water. All the slides were stained for 25 minutes using LEICA AUTO STAINER, XL, with Ehrlich's haematoxylin and counterstained with eosin. Cover slips were attached to the slides with DPX mounting solution. DPX mountant for histology is a mixture of distyrene, plasticizer, and xylene (see Appendix I for details processing solutions, steps, and staining procedure).

The slides were viewed or examined using a light compound microscope (OLYMPUS CH-30). Using a computer programme NIS Elements BR 3.0 (Driver Nikon Ds-U1, version 5.547) the image was enlarged onto a monitor for analysis. Photomicrographs of images shown on the screen were taken with Nikkon Digital Sight, DS Fi1 camera mounted on Zeiss-OLYMPUS CH-30 microscope. On the NIS Elements BR 3.0 programme, the images were resized, sharpened and colour adjusted prior to photographing. The images were combined into plates labelled on Microsoft Office Power Point, 2010, using a free hand.

Histological data recorded included the presence or absence of gonadal damage, and preparations were graded according to a pre-determined scale, using the control samples as a base line. The percentage frequency of each grading on the numerical scale was tallied and recorded for analysis.

These criteria are based on certain consistent histopathological changes that occur in tilapia gonads in response to chemical exposure. The criteria are graded for severity on a numerical scale (Tables 3.6 and 3.7 in Chapter 3), as seen under the microscope in comparison with Morrison *et al.* (2006) and the control of this a study, using OECD (2009), as a guide for the description.

The respective preparations were scored according to the following guidelines:

For assigning a score of between 0 and 1, more than three traits have to be observed.

For assigning a score of between 1 and 2, more than 3 of traits (ii) to (vi) had to be observed.

To assign a score of between 2 and 3, more than 4 of the criteria had to be observed.

For assigning a score of between 3 and 4, more than 5 of the traits had to be observed.

4.2.6 Data analysis

Means of total length, total body weight, depth were computed using Microsoft Excel 2010 and Sharp Model ELW53H Scientific Calculator). A one-way analysis of variance (ANOVA) was performed to test for the significance of variance for the weight, length and depth traits between different treatments using SAS

Version 9.1. The GSI values were subjected to logarithmic transformation to stabilise the variance to obtain a normal distribution. Correlation between the treatment concentration (of the diets) effect on the gonadal development (i.e. GSI) of the fish were estimated, by one-way ANOVA (using SAS Version 9.1), pairwise t-test by the Least Square Means (LSM) at significance level of 95% ($P<0.05$). Due to the smalls sample size (n=5) per treatment all the fish (both male and female) were pooled together according to diet (Pawpaw and Moringa) for the analysis as required by the programme.

The effects of treatments on the gonadal development were graded according to a numerical scale, that was developed based on aset of diagnostic criteria depicting observation of the histopathology of fish gonads. The criteria for the grading were set to be flexible enough to accommodate the various tissue changes, as proposed by OECD (2009).

4.3 Results

4.3.1 Biological: Body and Organ Measurements

The average body total length, weight, depth and coefficient of variation in the weights per treatments for both male and females were computed and presented in Tables 4.3. and 4.4.

Table 4.3 Body characteristics (means ±se) of sexually mature male Mozambique tilapia (*Oreochromis mossambicus*) fed diets containing different levels of Pawpaw and Moringa seed meal during a 60 day treatment period.

Treatment	N	Length (mm)	Weight (g)	CV Weight (%)	Depth(mm)	GSI
Control (C)	5	$156.60^{a} \pm 2.46$	$62.45^{b} \pm 3.11$	11.0	$42.00^{a} \pm 0.85$	$0.66^{a} \pm 0.11$
Pawpaw						
P0.5	5	$161.20^{b} \pm 2.36$	$64.90^{b} \pm 3.52$	12.1	$43.20^{a} \pm 1.25$	$0.61^{a} \pm 0.86$
P1.0	5	$162.20^{b} \pm 1.89$	$66.97^{b} \pm 2.54$	8.5	$43.80^{a} \pm 1.14$	$0.69^{a} \pm 0.12$
P5.0	5	$149.00^{a} \pm 2.99$	$54.96^{a} \pm 4.39$	17.5	$39.60^{a} \pm 1.45$	$0.72^{b} \pm 0.07$
P10.0	5	$162.00^{b} \pm 2.95$	$65.15^{b} \pm 4.76$	16.3	$41.00^{a} \pm 1.52$	$0.85^{b} \pm 0.05$
P15.0	5	$160.00^{b} \pm 3.48$	$62.05^{b} \pm 5.92$	21.2	$41.00^{a} \pm 1.72$	$0.64^{a} \pm 0.07$
Moringa						
M0.5	5	$160.50^{b} \pm 1.14$	$62.05^{b} \pm 1.29$	4.6	$41.80^{a} \pm 0.82$	$0.76^{b} \pm 0.07$
M1.0	5	$164.40^{b} \pm 1.91$	$65.57^{b} \pm 0.99$	3.4	$42.40^{a} \pm 1.28$	$0.80^{b} \pm 0.18$
M5.0	5	$161.00^{b} \pm 3.49$	$65.56^{b} \pm 4.38$	14.8	$43.20^{a} \pm 1.61$	$0.84^{b} \pm 0.06$
M10.0	5	$157.00^{a} \pm 3.62$	$61.22^{b} \pm 4.50$	16.4	$40.30^{a} \pm 1.59$	$0.81^{b} \pm 0.09$
M15.0	5	$156.20^{a} \pm 3.09$	$58.78^{a} \pm 3.21$	12.2	$39.40^{a} \pm 1.31$	$0.98^{b} \pm 0.11$

Differences between the control and experimental diets were verified at $p<0.05$ (pairwise, t-test using LSM). Values in the columns with same letter are not significantly different

The highest body weight was 66.97 ±2.54g recorded in P1.0. The mean values of body measurements total length (TL), body weight (BD) and depth (D) across the treatment groups were not significantly different, indicating that in spite of the huge size range (20-45g) of the fish, the distribution into the 70 tanks done randomly was normally distributed. The mean GSI between the sexes (i.e. M or F) was significantly different at P <0.001. The effect of Pawpaw and Moringa on GIS compared to the control was significantly different (*P*-value = 0.001), but between the Pawpaw and Moringa it was not significantly different. In terms of treatment effect on the sex (male and female), the treatment groups were significantly different at P<0.05 (*P*-value =0.001).

Table 4.4 Body characteristics (means ±se) of sexually mature female Mozambique tilapia (*Oreochromis mossambicus*) fed diets containing different levels of Pawpaw and Moringa seed meal during a 60 day treatment period

Treatment	N	Length (mm)	Weight (g)	CV Weight (%)	Depth (mm)	GSI
Control (C)	5	138.40^{a} ± 3.33	42.87^{a} ± 3.33	17.9	35.00^{a} ± 0.98	4.25 ± 0.75
Pawpaw						
P0.5	5	141.80^{b} ± 2.07	48.36^{b} ± 3.34	15.6	37.20^{a} ± 1.04	5.64 ± 0.55
P1.0	5	135.20^{a} ± 2.90	42.08^{a} ± 1.77	9.4	35.50^{a} ± 1.04	4.97 ± 0.58
P5.0	5	136.50^{a} ± 3.86	42.26^{a} ± 3.04	14.3	36.00^{a} ± 1.14	4.16 ± 0.68
P10.0	5	133.75^{a} ± 2.84	40.33^{a} ± 2.70	14.9	34.75^{a} ± 1.77	2.85 ± 0.80
P15.0	5	138.60^{c} ± 1.99	43.51^{a} ± 1.43	7.4	34.60^{a} ± 67.7	5.72 ± 1.29
Moringa						
M0.5	5	140.00^{b} ±2.82	44.83^{c} ± 2.26	11.3	35.80^{a} ± 0.77	5.77±0.48
M1.0	5	135.6^{a} ± 4.02	42.73^{a} ±4.10	21.4	36.00^{a} ±0.60	4.25±0.86
M5.0	5	137.43^{a} ± 2.01	44.26^{b} ± 2.49	12.6	35.60^{a} ± 0.82	5.97±0.61
M10.0	5	132.21^{a} ±1.3 1	38.03^{a} ±1.10	6.2	33.20^{a} ± 0.82	5.84±0.32
M15.0	5	142.0^{b} ± 4.11	44.20^{b} ± 2.89	14.56	34.60^{a} ±0.61	6.16±0.94

Differences between the control and experimental diets were verified at p<0.05 (pairwise, t-test using LSM). Values in the columns with same letter are not significantly different.

In the Pawpaw fed fish, the largest mean weight of females fish, was obtained in M0.5.0g/kg BD 48.97 ± 3.34 and mean length 141.80 ± 2.07. The lowest was obtained in M10.0g/kg BD, weight 40.33 ± 2.70 and length 133.75 ± 2.70. In the Moringa fed fish, the largest mean weight was obtained in M0.5g/kg BD 44.83 ± 2.26 and mean length 140.00 ± 2.82.The lowest was obtained M10.0g/kg BD, weight 38.03 ± 1.21 and length 132.21 ± 1.31.

In the Pawpaw fed fish, the largest mean weight was obtained in P1.0g/kg BD 66.97 ± 2.54 and mean length 162.20 ±1.89. The lowest was obtained P5.0g/kg BD, weight 54.96 ± 4.39 and length 149.00 ± 2.99. In the Moringa fed fish, the largest mean weight was obtained in M1.0g/kg BD 65.57 ± 0.99 and mean length 164.40 ± 1.91.The lowest was obtained P10.0g/kg BD, weight 58.78 ± 3.21 and length 156.20 ± 3.09.

4.3.2 Histological Evaluation

The severity levels of the gonadal damage in the fish in experiments I.a and I.b are presented in Tables 4.5 and Table 4.6 respectively. The highest incidence of pronounced severity of 60% (n=5) was induced in Moringa at 10.0 and 15.0 g/kg basal diet in the males. No apparent abnormal oocyte growth and development such as presence of atretic oocytes were seen in the ovaries of most of the females.

Table 4.5 The influence of different inclusion levels (g/kg BD) of Pawpaw seed meal (P) and Moringa seed meal (M) in a commercial tilapia diet on the gonadal integrity of sexually mature male and female Mozambique tilapia (*Oreochromis mossambicus*; 20-45g) during a treatment period of 60 days.

Treatment	N	Observed degree of gonadal damage per gender							
		No effect (normal)		Minimal Effect		Moderate effect		Pronounced effect	
		♂	♀	♂	♀	♂	♀	♂	♀
Control (C)	10	50	50	-	-	-	-	-	-
Pawpaw									
P0.5	10	50	10	-	40	-	-	-	-
P1.0	10	40	10	10	40	-	-	-	-
P5.0	10	10	10	30	30	10	10	-	-
P10.0	10	-	10	-	30	30	-	30*	
P15.0	10	-	10	10	40	30	-	10	-
Moringa									
M0.1	10	20	10	30	40	-	-	-	-
M1.0	10	10	-	30	30	10	20	-	-
M5.0	10	-	-	10	10	30	40	10	-
M10.0	10	-	-	-	30	10	20	40*	-
M15.0	10	-	-	-	10	30	40	20	

*Egg producing tissue i.e. testis-ova observed.

In Experiment I.b, different combinations of Pawpaw and Moringa were fed to sexually mature Mozambique tilapia to determine any synergistic effect of the two endocrine disrupting compounds. The results are presented in Table 4.6.

Table 4.6 The influence of 17-methyltestosterone (MT) and different combinations of Pawpaw seed meal (P) and Moringa seed meal (M) included in a commercial tilapia diet on the gonadal integrity of sexually mature male and female Mozambique tilapia (*Oreochromis mossambicus*; 20-45g) during a treatment period of 60 days.

Treatment	N	Observed degree of gonadal damage per gender (%)							
		No effect (normal)		Minimal effect		Moderate effect		Pronounced effect	
		♂	♀	♂	♀	♂	♀	♂	♀
Control diet (BD)	10	50	50	-	-	-	-	-	-
MT (60mg/kg BD)	10		30	10	10	40	10	-	-
10g P + 5g M /kg BD	10		10	-	30	20	10	30	-
5g P + 10g M /kg BD	10		10	10	30	20	10	20	

Incidence of pronounced severity was induced in both P10M5 and P5M10 g/kg basal diet in the males, no such state was found in 17MT. No apparent abnormal oocyte growth and development such as presence of atretic oocytes were seen in the ovaries of most of the females

The effect of Pawpaw and Moringa seed meal on the gonadal integrity is shown in Plate 1.Common disruption of the gonads observed were the degeneration of the seminiferous tubules found at the higher inclusion levels (i.e. 5.0, 10.0 and 15.0g/kg Basal Diet) of both Pawpaw and Moringa. A testis with egg producing cells was found in the male gonads of tilapia fed with 10.0g/kg basal diet of both Pawpaw and Moringa seed powder (Plates 4.1)

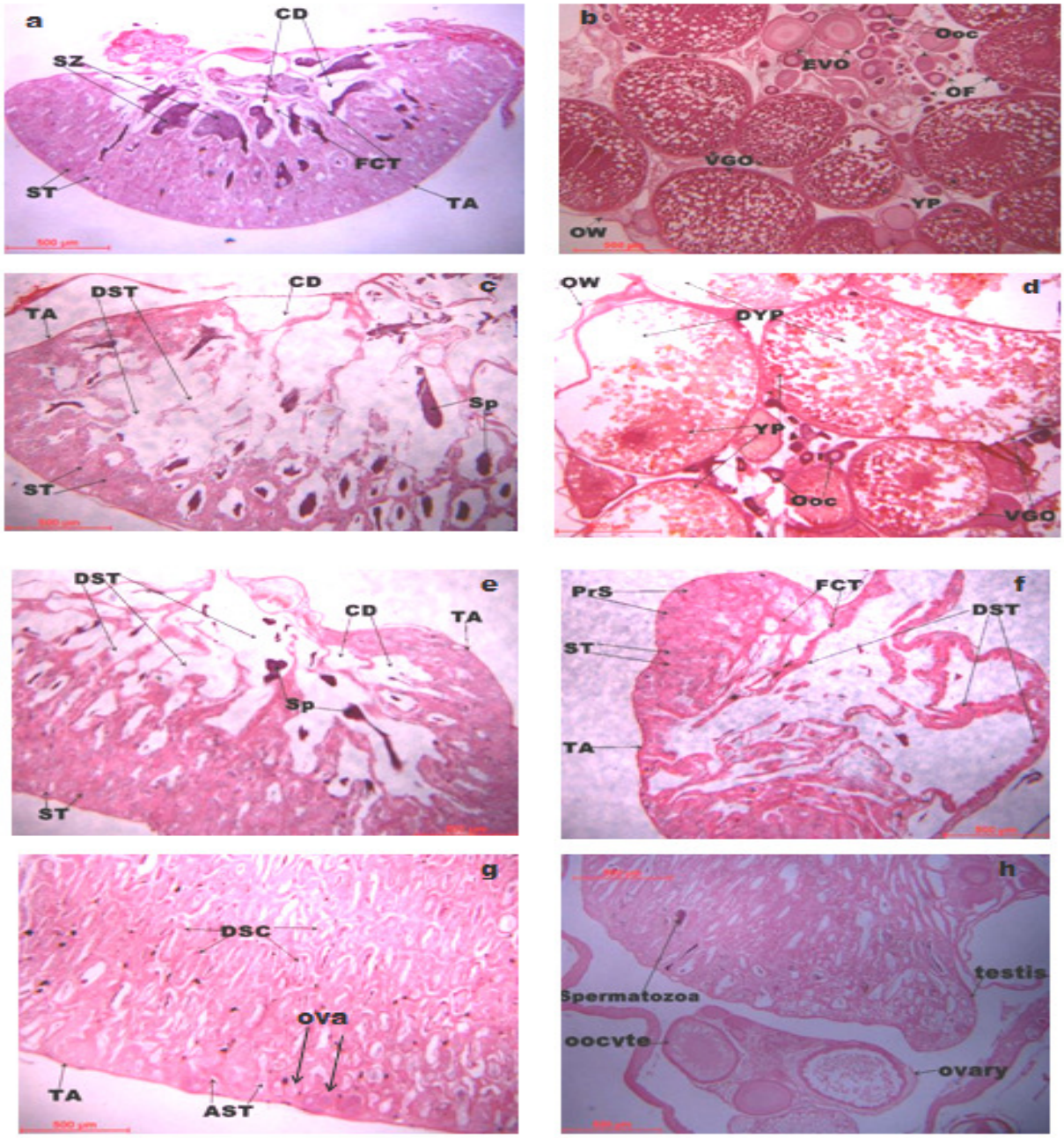

Plate 4.1 The influence of Pawpaw seed meal and Moringa seed meal included in a commercial tilapia diet (basal diet, BD) at 0, 0.5, 1.0, 5.0,10.0 and 15.0g/kg basal diet respectively, on the gonadal integrity of sexually mature Mozambique tilapia (*Oreochromis mossambicus*, 20-45g) during a 60 day treatment period.

a) Normal testis (BD); b) Normal ovary (0g/kg BD); c) Testis of fish receiving 5.0g M/kg BD; d) Ovary of fish receiving 10.0g M/kg BD; e) Testis of fish receiving 5.0g P/kg BD f) Testis of fish receiving 10.0g P/kg BD; g) Testis-ovarian tissue observed in fish receiving 10.0g M/kg BD; h) Testis-ovarian tissue receiving 10.0g P/kg BD.

AST = atrophied seminiferous tubules; CD = collecting duct; DSC = disintegrating sperm cells; DST = degenerating seminiferous tubules; DYP = depleting yolk particles; EVO = early developing oocytes; FCT = fibrous connective tissue; OF = ovigerous follicle; Ooc = developing oocytes; OW = ovarian wall; PrS = Primary spermatogonia; Sp = spermatids; ST = seminiferous tubules; SZ = spermatozoa; TA = tunica albuginea; VGO = vitellogenic oocytes; YP = yolk particles. Bar = 500µm.

4.4 Discussion.

4.4.1 Influence of Pawpaw and Moringa on Biological Characteristics

The majority of cultured fish exhibited a gonochoristic type of sexual differentiation, i.e. either testes or ovaries are observed in an individual (Yamazaki, 1983; Nagahama, 1994). Both the ovaries and testes of teleost fish are paired, and are usually equal in size. Paired gonads were observed in male and female fish sampled from all the respective treatment groups, indicating that *O. mossambicus* is a gonochoristic fish. No deformities i.e. enlarged or twisted gonads were observed in the fish obtained from all the treatment groups. They are composed of follicles derived from the germinal epithelium and contain oogonia that develop into oocytes and ultimately ova, and the surrounding follicular epithelium. Also present are other supporting structures which include stroma, vascular and nervous tissues (Nagahama, 1994; Blazer, 2002). Tyler and Sumpter (1996) explained that the functional unit of the ovary is called the ovarian follicle, which refers generally to an oocyte plus its surrounding sheath of perifollicular cells (granulosa cells, theca cells, and the surface epithelium cells). The testes of most teleosts have two lobes composed of numerous lobules. These lobules, are separated by a thin layer of connective tissue. Leydig cells, involved in male hormone production, are a typical component of the interstitium. The developing germ cells include spermatogonia, spermatocytes, spermatids and spermatozoa (Nagahama,1994; Blazer, 2002). In comparison with the observation made, visually the gonads conform to what is considered as perfect gonads in gonochoristic fishes capable of producing viable gametes.

The result obtained indicates that gonadosomatic index (GSI) was influenced significantly (P <0.001) by the Pawpaw and Moringa particularly at higher inclusion levels. In the same vein the GSI seen in the interaction of the Pawpaw and Moringa treatment and 17α-methyltestosterone was affected (P <0.001). Generally, the mouth-brooding *Oreochromis* species such as *O. mossambicus* exhibit gonadosomatic index (GIS) between 4.6% and 10.2% (Peters, 1983). Coward and Bromage (2000) also reported that GSI of substrate-spawner *Tilapia zillii* raised in ponds reached 6.2%. In this study mean GSI for Control was 2.74% (n=10), Pawpaw was 3.11%, (n=47) and Moringa 5.09% (n=50). Moringa was significantly different from Pawpaw and Control at p<0.05 (P = 0.231). The fish used were sexually matured (20-45g), which reproduced during the experimental period, thus had the capacity to reproduce. The experiment was conducted at a time where in the wild it is the breeding season of tilapias of Southern Africa (Skelton, 2001). In the experiment the temperature was maintained within the range favourable for tilapia to reproduce (i.e. 28^{o}C ± 2). Therefore, the GIS observed indicate that the fish was ready to spawn. The experiment was not designed to look at the suppression of reproduction by the plant materials., However, the reproductive activity of the fish was monitored during the study period, in terms of brooding female, which occurred frequently in the control and low levels of Pawpaw and Moringa (0.5, and 1.0). This observation is explained vividly by the histological observation as shown in Plate 4.1 and explained in Section 4.4.2.

4.4.2 Histological Assessment of Gonadal Damage Severity

A set of diagnostic criteria was created to evaluate any form of abnormalities observed in the gonads during this study. Based on the criteria four main severity level on a numerical scale from 0 to 4 was established as

none (0-1), low (1-2), moderate (2-3) and pronounced (3-4). A gonadal section was assigned to any of the grade by observing or identifying some number of the description presented in Table 3 (Chapter 3, section 3.6.5). Gonadal histological evaluations or analysis can provide insight into the threats or effects presented by environmental stressors on reproductive health of fishes. In fact one of the most powerful tools available to conduct assessment of endocrine-disrupting effects on fish has been found to be histopathology (Van Der Ven *et al*., 2003), and therefore it is used in the study of reproductive health of fishes (Blazer, 2002). Histopathology has been described as a descriptive and interpretive science, accordingly it is rather subjective. However, histopathological evaluations of the same study by any qualified pathologist should identify the same treatment-related findings (Crissman *et al*., 2004, USEPA, 2006; OECD, 2009). In fact fish gonadal state and reproduction are now considered to be the more reliable indictors of endocrine disruption in aquatic systems by both natural and artificial chemicals (Jobling *et al,* 1998; Celino *et al*., 2009). Therefore, the grading system was created with the view that it could be comparable and consistent with that of USEPA (2006) and OECD (2009).

The common disruptions of the male gonads encountered in this study were (i) degeneration of the seminiferous tubules (ii) disintegration of the germ cells and (iii) testis-ova (Plates 4.1 e, g & h). These were observed in the higher inclusion levels of 5.0, 10.0, and 15.0 g/kg basal diet of both Pawpaw and Moringa. The testis-ova (a testis with egg producing cells) was found in a male gonad of tilapia fed with 10.0g/kg basal diet of both Pawpaw and Moringa seed powder. In the female gonads, the common changes observed were (i) oocyte atresia and (ii) depletion of yolk particles (see Appendix I). Pronounced severity on integrity of the gonad was observed in P5.0, P10.0, P15.0, M5.0, M10.0 and M15.0 which were not significantly different at $p<0.05$, but significant compared to the other inclusion levels at $p<0.05$ ($P = 0.0366$). However, few atretic oocytes were observed in fish fed P5.0g/kg and M5.0, M10.0 and P or M15.0g/kg and classified as moderate (Table 4.3).

Evidence available suggests that the incidence of testis-ova (intersex, or ovotestis) is linked with discharges of estrogenic substances into the aquatic environment and this change has been suggested as a marker for exposure to these substances (Jobling *et al*., 1998; Pait and Nelson, 2002; Manning, 2005). Both USEPA (2006) and OECD (2009) guidelines for the diagnosis of endocrine-related histopathology of fish gonads emphasise that the primary criteria of interest for diagnosis include (i) presence of testis-ova, (ii) increased proportion of spermatogonia, (iii) increased testicular degeneration and (iv) interstitial (Leydig cell) herperplasia/hypertrophy. Likewise, in the female gonad the following are the primary criteria (i) increased oocyte atresia, (ii) perifollicular cell hyperplasia/hypertrophy, (iii) decreased yolk formation and (iv) changes in gonadal staging (i.e. insufficient tissue, extensive necrosis, inflammation and artefact).

In comparison with respect to the primary guidelines of USEPA (2006) and OECD (2009), both Pawpaw and Moringa seed fed to Mozambique tilapia (*Oreochromis* mossambicus), elicited at least 2 effects in this study in each sex. For instance the male gonads showed (i) the presence of testis-ova, (ii) an increased proportion of spermatogonia and (iii) increased testicular degeneration. Also in the female gonad the commonest effects were (i) increased oocyte atresia, (iii) decreased yolk formation or yolk depletion and (iii) changes in

gonadal staging. In the females one of the major problems was that most of the gonads could not properly or reasonably be staged because there appeared to be insufficient tissue and artefacts (USEPA, 2006; OECD, 2009), so in some of the females the ovary showed eggs with empty ova (Appendix I).

Studies both from the field and laboratory experimentations have established that in fish continuous exposure to endocrine disrupting chemicals (EDCs) leads to alteration of their reproductive physiology and morphology which include changes in or skewed sex ratios,higher incidences of reproductive defects in larvae, egg-producing cells in the male testis (ovo-testis), reduced gonadal (i.e. testis and ovary) growth rates and size, reduced female reproductive tracts, feminisation of reproductive ducts, reduced gamete quality, degeneration of testicular germinal cells, increased liver size, increased levels of and vitellogenin (egg-yolk protein) in male blood plasma (Jobling *et al*., 1998; Mills and Chichester, 2005; Nash *et al*., 2006; Vajda *et al*., 2011). In this study, egg-producing cells in the male testis (i.e. testis-ovo) and degeneration of testicular germinal cells were observed (Plate 4.1c, e, f, g & h).

Among EDCs that find their way into the aquatic environment are naturally occurring chemicals, human and animal wastes products (e.g. estrogen), which could be steroidal (such as 17 β-estradiol, estrone and estriol) or non-steroidal. Medicinal supplements, such as those recommended for estrogen replacement therapy in post-menopausal women, contain high levels of phytoestrogens (Sassi-Messai *et al*, 2009; Vadja *et al*., 2011; Kosai *et al*., 2011). Industrial activities such as pulp and paper production also can release large quantities of phytoestrogens which have been found to affect reproductive system function in fish. Pait and Nelson (2002) indicated that one major by-product that comes out of pulp and paper mill is β-sitosterol (a major plant sterol), which is linked to induced production and increased levels of vitellogenin in fishes. Shanhan *et al*., (2009) reported that phytoestrogens show various effects on the reproduction of both male and female fishes in water bodies where they abound. The degradation of vegetable matter and paper products in wastewater treatment plants also contribute to releases of phytoestrogens into the aquatic ecosystem (Takurah and Takemura, 2011).

The earliest report of phytoestrogens as endocrine disrupters in fish came to light from the works of Pelissero *et al*. (1991) and Bennetau-Pelissero *et al.* (1998). The most common phytoestrogen found in majority of edible plants are flavonoids (flavonols, isoflavonoids, and isoflavans); coumestans (coumestrol); lignans; saponins and the triterpenes. The most common isoflavonoids are genistein and diadzein, and the flavonoids are quercetin and kaempherol, which are widely distributed in the Plant Kingdom (Mazur, 2000; Patisaul and Jefferson, 2010). Since the estrogenic activity of flavonoids in fish diets was reported by Pelissero *et al,* (1991), some laboratory studies have been conducted using genistein and equol (a by-product from intestinal digestion of diadzein). For instance, Kiparissis *et al.* (2003) treated the Japanese medeka (*Oryzias latipes*) with genistein (1,000µg/L) and equol (0.4 and 0.8µg/L) from soon after hatch to approximately 100 days posthatch induced testis-ova in males and delayed oocyte maturation and atretic oocytes in females. Kosai *et al.* (2011) treated Nile tilapia with 10 and 100µgL^{-1} of 17β-estradiol for 30 days and found that the high level group showed testes-ova in the males. The authors concluded that 17β-estradiol at high concentrations positively changes male reproductive system in Nile tilapia.

Phytoestrogens are found in a variety of plants, but are most abundant in leguminous plants and whole grain cereals (Kurzer and Xu, 1997; Mazur *et al,* 1998), which also form the bulk of basic ingredients in manufacturing of aquaculture feeds ingredients (El-Sayed, 2006; Rana *et al*, 2009). The reported phytoestrogens identified in Pawpaw plant parts include β-sitosterol, saponins, and flavonoids (Krishna *et al.*, 2008) and that of Moringa plant parts include saponins, triterpenes, e.g. oleanolic acid-3-glucoside and flavonoids -quercetin and kaempferol (Anwar *et al.*, 2007; Kumar *et al.,* 2010). In a review by Kumar *et al.*, (2012), the authors indicated that oleanolic acid-3-glucoside and β-sitosterol are some of the phytochemicals that have shown 100% antifertility activities, which are present in the Moringa plant. These phytochemicals in Pawpaw and Moringa make them potential endocrine disrupters.

According to Segner (2011) the physiological regulation of reproduction in sexually mature fish integrates information from environmental cues (e.g., water temperature and photoperiod), external stimuli (e.g. mating behaviour) with internal signals (e.g., nutritional status) to promote or suppress the steps of gonad maturation, spermatogenesis/oogenesis, reproductive behavior and spawning. Available literature indicates that there is no clear cut mechanism by which a phytoestrogen elicit its effect as an endocrine –disrupter. In other words the exact mechanism by which phytoestrogens affect the physiological activity of fish is unknown. However, Moutsautsou (2007) intimated that the likely route is through binding with the estrogen receptors (ERs), changing the metabolism of endogenous steroid hormone and modulating the function of enzymes.

Field and laboratory studies over time have informed scientists to believe that EDCs mode of action is employed through one or a combination of these routes which include 1) simulating the effects of endogenous hormones (i.e. estrogens and androgens; 2) blocking the effects of endogenous hormones; 3) changing patterns of synthesis and metabolism of normal hormones; 4) through modification of hormone receptor levels, and also 5) interfering with the binding proteins transporting endogenous hormones to the target tissues (Jobling *et al*., 1998; Pait and Nelson, 2002; Mills and Chichester, 2005; Lyssimachou, 2008). Thus, endocrine disruption results from exogenous compounds interaction with the internal endocrine signalling pathways in an organism (Mills and Chichester, 2005).

Information or literature on Pawpaw and Moringa as potential endocrine disrupters (Evans *et al.,* 2011), is scanty. Few attempts have been made to check their effect on reproduction in tilapia (Ekanem and Okoronkwo, 2003; Jegede and Fagbenro, 2008; Abbas and Abbas, 2011). All these authors adduced that, Das (1980) mentioned oleanolic glycoside as an active component of Pawpaw seed, which had been found to cause sterility in male rats. However, according to Das (1980), citing Fransworth (1975), the active principle responsible for anti-implantation effect of papaya seed in female rats, might be 5-hydroxytryptamine. In the case of Moringa, the reported blocking of reproductive activity has been manifestation of antifertility activity in rats. Ethanolic and aqueous extract of *M. oleifera* roots possess estrogenic, anti-estrogenic, progestational and antiprogestational activities in rats. Aqueous extracts of the root and the bark of *M. oleifera* are effective in preventing implantation in rats (Prakesh *et al*., 1985; Shukla *et al*., 1988; Sethi *et al*., 1988; Shukla *et al*., 1989; Kumar *et al*., 2010; Kumar *et al*., 2012).

Available literature indicates that there is no clear cut mechanism by which a phytoestrogen elicits its effect as an endocrine disrupter. However, various studies both in the field (e.g. Jobling *et al,* 1998; Blazer, 2002) and laboratory (Kiparissis *et al,* 2003) seem to suggest that phytoestrogens particularly, genistein, mimic the natural hormone estrogen. Phytoestrogens as estrogen-like compounds, may affect the activities of germ cells and the level of sex hormone, and disrupt sex differentiation thereby may induce vitellognin production in both female and male fish. Latonnella *et al.* (2002) and Moutsautsou (2007) suggest that phytoestrogen effects are mediated through the estrogen receptor (ER) subtypes alpha ERα and beta ERβ, which have been demonstrated to be cell type/tissue specific and dose-dependent Thus, phytoestrogens can bind to steroid-binding proteins and to estrogen receptors (ER) of target cells, mimicking the effects of endogenous hormones thereby blocking their effect in a female gonad stopping vitellogenin accumulation in eggs and also testicular cell proliferation. This could lead to oocyte atresia (i.e. the degenerative and reabsorption process particularly vitellogenic eggs in normal physiological process) and sperm necrosis (i.e. unprogrammed cell death of various stages of germ cells within spermatogenic cycle). Sperm necrosis and cell syncytia (fusion of cells), have been reported after exposure to natural and synthetic estrogenic compounds (Blazer, 2002). Maclatchy and Van Der Kraak (1995) also indicated that phytoestrogen manifest endocrine-disturbing activity by interfering with enzymatic reactions either on steroid metabolism (i.e. aromatization) or on the mechanism of action of estrogens (i.e. tyrosine kinase activity). In view of this phytoestrogens can induce reproductive disorders.

In the microphotographs obtained in this study in which testes-ova and degeneration of germ cells were observed showed the deformities in a single gonad structure suggesting that phytoestrogen activity does not follow a single pathway. It appears the mechanism involves multiple (two or more) EDCs in action (Pait and Nelson, 2002; Mills and Chichester, 2005; Lyssimachou, 2008).

4.5 Conclusion

In this study, feeding sexually mature (20-45g) Mozambique tilapia (*Oreochromis mossambicus*) at hgher inclusion levels of 5.0, 10.0, and 15.0 g/kg BD of Pawpaw and/or Moringa Seed powder produces in males (i) degeneration of the seminiferous tubules (ii) disintegration of the germ cells and (iii) testes-ova (Figures 4.2e, g & h), and in the female gonads, (i) atretic oocyte and (ii) depletion of yolk particles. It has been established that Pawpaw contains phytochemicals β-sitosterol, saponins, and flavonoids (Krishna *et al*, 2008), and Moringa contains saponins, triterpenes, e.g. oleanolic acid-3-glucoside and flavonoids (quercetin and kaempferol) (Anwar *et al*., 2007; Kumar *et al,* 2010), β-sitosterol (Kumar *et al*, 2012), and oleanolic acid in this study. These observation wihch are consistent with other field observations and laboratory studies such as that of Pelissero *et al.* (1991), Maclatchy *et al.* (1995), Jobling *et al.* (1998), Blazer (2002), Latonnelle *et al.* (2002), Kiparissis *et al.* (2003), Mills and Chichester (2005), and Sassi-Messai *et al.* (2009), clearly show that Pawpaw and Moringa affect the gonadal integrity of Mozambique tilapia (*Oreochromis mossambicus*). In this vein Pawpaw and Moringa could be considered as potential endocrine disrupters (Evans *et al,* 2011), and as such there is a window of opportunity or possibility for the use of these plants to manipulate reproduction in tilapias.

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Chapter 5

The Effect of Pawpaw Seed Meal and Moringa Seed Meal on Gonad Development and Function of Sexually Immature Mozambique Tilapia (*Oreochromis mossambicus*)

Abstract

In Sub-Sahara African (SSA) countries, culture efforts of tilapia are hampered by restricted access to appropriate methods to control the precocious breeding strategy. Uncontrolled breeding leads to overstocking in culture systems, resulting in stunted growth and a variation in marketable size of the fish. Alternative methods that will provide suitable and cost-effective options to resource-poor communities to culture tilapia are needed to ensure the viability and sustainability of tilapia production, especially in SSA. A potential method to control the precocious breeding nature of tilapia is the use of natural plants components that contain endocrine disrupting chemicals. Endocrine disrupting chemicals mimic the behaviour of the natural reproductive hormones, but negatively affect the function and integrity of the gonads and related glands. Several studies report on the anti-fertility effect of Pawpaw (*Carica papaya*) and Moringa (*Moringa oleifera*) seeds in certain livestock species. This study investigated the potential of Pawpaw seed meal (P) and Moringa seed meal (M) as feed supplements to control the gonadal differentiation in sexually immature Mozambique tilapia (*Oreochromis mossambicus*). A total of 1650 sexually immature tilapia (with unknown sex) weighing between 2-8g, were randomly allocated to 55 tanks at a stocking density of 30 fish per tank. After an acclimatization period of 7 days, fish were fed the respective experimental diets containing 0, 0.5, 1.0, 2.0, 5.0 and 10.0 g of either P or M per kilogram feed, respectively. The experimental diets were fed for a period of 60 days, after which 4 fish were sacrificed per treatment to assess the effects of P and M on gonadal function and integrity. No lesions were observed in fish receiving the control diet (0g/kg feed). Treatment diets containing 2.0, 5.0 and 10.0g/kg feed P of M resulted in atrophy of the seminiferous tubules in male fish, as well as disintegration of sperm cells and necrosis of testicular cells. Testis-ovarian tissue was observed in the groups that received 10.0g P or M per kilogram feed. In the females, atresia of oocytes was observed in 10.0 g/kg basal diet for both P and M. The study indicated that P and M at inclusion levels of 2.0g/kg feed and higher have a significant anti-fertility effect, affecting gonad integrity and preventing reproduction in sexually immature male and female *O. mossambicus*. Pawpaw seed meal and Moringa seed meal can thus, be used as potential endocrine disrupting feed supplements to suppress the attainment of sexual maturity in tilapia, and thus control precocious breeding of tilapia in culture systems. Sustainable availability of Pawpaw and Moringa seeds in SSA countries contributes to the attractiveness of the two feed supplements to be used in the reproductive management of tilapia in commercial and small-scale tilapia culture systems.

5.1 Introduction

Tilapias have become the most important group of aquaculture species, second to carp, with an annual world production of 2.5 million tonnes. Tilapia species gained popularity as a food species after their introduction to tropical, subtropical and temperate regions all over the world, being cultivated primarily for food, recreational use, aquatic weed control, and research purposes (Pilay, 1990; El-Sayed, 2006). According to the Food and Agricultural Organization (FAO; 2012), tilapia production in 2010 was estimated

at 3.5 metric tonnes valued at US$ 5.7 billion (ZAR 50.73 billion). Currently tilapia is cultivated in almost 100 countries worldwide with over 98% of tilapia production outside their original habitats of Africa (Shelton and Popma, 2006; FAO, 2012). The drastic increase in tilapia culture can be ascribed to the advantageous aquaculture traits of two tilapia species, the Nile tilapia (*Oreochromis niloticus*) and the Blue tilapia (*O. aureus*), and various hybrid combinations of these species with the Mozambique tilapia (*O. mossambicus*) (FAO, 2010). The Mozambique tilapia has been a major contributor to the various hybrid tilapia populations around the world, particularly the red tilapias (Campos-Ramos *et al*, 2003). Characteristics like a fast growth rate, ease of adaptation to a wide range of environmental conditions, ready acceptance of artificial feed, and ease of production, all contribute to *Oreochromis* species being favoured as aquaculture food species.

The FAO (2011) report on the state of international food insecurity indicated that the majority of the countries that were hit by food and economic crisis during 2006 to 2008 are located in sub-Saharan Africa (SSA) countries such as Ghana and Malawi. The fact that the majority of the SSA countries affected by the recession are poor and food importers, necessitate a multi-faceted approach to alleviate poverty and increase food security. The World Fish Centre (WFC; 2005) identified fish as one of the key vehicles to ensure food security and safety, and maintains that fisheries have been demonstrated to be able to improve the household security of SSA nations, distributing the benefits widely and cost-effectively among women and the poor, thus making a unique and vital contribution to human capital development.

In sub-Saharan Africa (SSA), fish provides up to half of animal protein for human consumption and in 15 African countries, fish still represent over 30% of animal protein consumption. These countries include Uganda (31.6%), Tanzania (32.8%), Guinea (34.9%), Angola (35.7%), Côte d'Ivoire (36.0%), Togo (39.7%), Nigeria (40%), Malawi (44.2%), Congo (45.3%), Senegal (47-62%), Gambia (47.3%-62%), Cameroon (49%), Equatorial Guinea (58.2%), Sierra Leone (66.4%), and Ghana (68.6%). Most of SSA, tilapia production comes from capture fisheries in the extensive freshwater bodies such as Lake Volta in Ghana, the Rift Valley Lakes-Victoria, Tangayika and Albert of East Africa, with Africa as the top most world producer, Egypt and Uganda as the leading countries (El-Sayed, 2006). Despite the low contribution (1%) of SSA aquaculture to world aquaculture production, it is generally believed that aquaculture has the potential to provide the necessary benefits for the livelihoods of the poor either through an improved food supply and/or through employment and increased income (Moehl *et al.*, 2005; Brummett *et al.*, 2008; FAO, 2012). Although aquaculture is a recent activity in SSA, its yields increased there by 13% from 1970 to 2008.

The genus *Oreochromis*, in particular *O. niloticus, O. mossambicus* and *O. aureus,* has dominated tilapia cultivation worldwide. These species are extremely adaptable, and have the ability to cope with a variety of adverse environmental and management conditions. However, the main drawback in tilapia culture is the early sexual maturation of females at a very small size (i.e. 15-30g), which leads to precocious breeding and the resulting overpopulation of production systems. This overpopulation results in stunted growth, and a longer interval to reach a marketable size due to a large variation in size (Varadaraj and Pandian, 1987; Mair and Little, 1991; Popma and Lovshin, 1996). Stunting in tilapias is a huge problem for mixed sex culture and is partly the reason for the absence and low popularity of commercial aquaculture in SSA.

Several methods have been developed to curb this phenomenon of early puberty and sexual maturity in tilapia, with monosex culture of all-male populations being the preferred method. Pandian and Varadaraj (1990) reported that the four main methods to produce monosex tilapia populations include manual sexing of fingerlings and separation of the sexes; hybridization; sex reversal by hormone treatment, and chromosome manipulation. Mair and Little (1991) also enumerated the various methods and techniques available for the control of prolific breeding in tilapia, however, each of the techniques has its own limitations. The most common method of producing monosex all-male tilapia is by oral administration of 17α-methyltestosterone (MT), and this hormone is used extensively in tilapia producing countries such as China and Egypt (Nagahama, 1994; Okuzawa, 2002; El-Sayed, 2006; Phelps, 2006; Taranger e*t al.,* 2010; El-Sayed *et al*, 2012) to manage the precocious breeding nature of tilapia. In teleost fish, puberty commences after gonadal sex differentiation, followed by the onset of spermatogenesis in males and vitellogenic ovarian development in females (Okuzawa, 2002; Patiño and Sullivan 2002).

Pifferrer (2001) reported that the sex steroids and their actions are involved in the process of sex differentiation in fish. During sexual differentiation, the sex steroids act mainly as morphogenic factors, and during sexual maturation they act as activation factors. Generally, in fish the timing of onset of puberty is determined by several external and internal factors including photoperiod, water temperature, food availability, somatic growth and gonadal status, all of which can be manipulated under farm conditions. Both endogenous and exogenous steroid hormones are considered to have a positive effect on pubertal development. It has been proposed that sex steroids are the natural inducers of onset of puberty in some male fish. For instance, in juvenile male African catfish, long-term treatment of 11ketotestosterone or its precursor stimulated testicular growth and spermatogenesis, probably through direct effect on the testes (Cavaco *et al*., 1998; Schulz and Goos, 1999). In the same vein environmental conditions such as water temperature and photoperiod can be effectively manipulated to determine the timing of puberty (Okuzawa, 2002), as has been done in salmonids (Taranger *et al*., 2010). Attainment of sexual maturity in tilapia, on the other hand, is a function of age, size, and environmental conditions. *Oreochromis mossambicus*, in general, reaches sexual maturity or puberty at a smaller size and younger age than *O. niloticus* and *O. aureus*. It has been found that tilapia populations in large lakes mature at a later age and a larger size than the same species produced in culture ponds. For example, *O. niloticus* matures at about 10 to 12 months and 350 to 500 g in several East African lakes. The same population under conditions of near maximum growth will reach sexual maturity in farm ponds at an age of 5 to 6 months and 150 to 200 g (Shelton and Popma, 2006). Thus, the assumption is that sexual maturation is delayed in stable habitats such as large lakes and dams, with precocious breeding occurring in unstable and shallow environments such as aquaculture pond systems. The ability to control the environment and hormone production in tilapia can be used to manipulate and/or suppress early puberty in tilapia.

Okuzawa (2002) emphasised that the end of puberty is indicated by the successful production of the first batch of fertile gametes, resulting in spermiation and sperm hydration in males, and ovulation in females. Extending the puberty period can potentially limit the attainment of sexual maturity, and the resulting proliferation of farmed fish. The steroid estrogen has been found to be produced before and after ovarian

differentiation (Nagahama, 1983), and the inhibition of estrogen synthesis can thus potentially increase the proportion of males in culture systems. Male tilapia has a fast growth rate, and has a greater uniformity in size at harvest. The use and worldwide acceptance of the hormone 17-α methyltestosterone to produce all-male populations, however, is hampered by the fact that it is expensive and difficult to obtain in SSA countries. The use of hormones to manipulate fish reproduction also presents some environmental and public health concerns, and consequent apprehension among consumers.

As the search for a better solution to this problem continues, medicinal plants have the potential to be used in the reproductive management of tilapia. Previous studies have shown that phytochemicals (i.e. chemicals derived from a plant) occurring in medicinal plants have an estrogen-like biological activity (Turker and Takemura, 2011), and thus have the potential to be used to manipulate the onset of puberty in fishes. According to Soejato *et al.* (1978), a plant or its extract could conceivably be useful as an anti-fertility agent, when its use results in anti-implantation, disruption of oestrous cyclicity, and luteolytic activity. Medicinal plants have been successfully used to induce sterility in laboratory animals (Bodharkar *et al.,* 1974; Das, 1980). Endocrine disruption occurs through the interact of exogenous chemicals with internal endocrine signalling pathways of an organism. Endocrine-disrupting chemicals (EDCs) exert their effects by simulating endogenous hormones, acting as antagonists of natural hormones by changing the natural process of hormone synthesis, metabolism and modifying hormone receptor levels (Pait and Nelson, 2002; Mills and Chichester, 2005). The environmental chemicals that occur in aquatic environments are referred to as xenoestrogens, and may include ethinylestradiol-17α, nonylphenol and polychlorinated biphenyls (PCB), which all can act as an endogenous estrogen, which adversely affect reproduction in teleost fish.

Any substance that can interfere with the activity of either estrogens or androgens, can lead to defect in the reproductive health of the fish. There is considerable evidence which indicates that exposure of an organism to EDCs or natural hormones which interfere with the normal functioning of the endocrine system has the potential to affect the reproductive endocrine function and change reproductive development (Jobling *et al*, 1998; Damstra *et al*, 2002; Manning, 2005; Mills and Chichester, 2005; Cheshenko *et al*, 2008; Sassi-Messai *et al.;* 2009; Blazer *et al*., 2012).

The main classes of phytoestrogens include the isoflavones (genistein, daidzein, glycitein, equol and biochanin A), the lignans (enterolactone, enterodiol), the coumestanes (coumestrol), the flavonoids (quercetin, kaempferol), the stilbenes (resveratrol), saponins and the triterpenes (e.g. oleanolic acid and ursolic acid). These compounds are all polyphenols whose molecular structure may or may not be similar to, but functionally similar to the natural androgens and estrogens, particularly 17β-estradiol and show biological activity in human and other vertebrates (Mazur, 2000; Dixon, 2004; Turan, 2006; Moutsatsou, 2007; Turker and Takemura, 2011). These phytochemicals particularly, genistein, daidzein apigenin, quercetin, kaempferol and naringenin, have been found to exhibit estrogenic effect and, also activate estrogen-receptor-mediated signalling.

A primarily plant diet for example omnivorous tilapias in the wild, and the use of soybean in the diet of fish in captivity, may be a source of considerable amounts of phytoestrogens (Pelissero and Sumpter, 1992). The effect of phytoestrogens on fish were initially documented and / or studied in trout and sturgeon (Pelissero and Sumpter, 1991; Pelissero *et al.,* 1991; Pelissero *et al.,* 1996). Pelissero *et al* (1991) assessed the estrogenic activity in daidzein, genistein, equol and coumestrol by their induction of hepatic synthesis of vitellogenin secretion in yearling Siberian Sturgeon. The result indicated that coumestrol appeared to be the most potent compound, inducing the most vitellogenin secretion with the lowest dose administered. Kiparissis *et al.* (2003) exposed Japanese Medeka (*Oryzias latipes*) to equol (a product of intestinal digestion of diadzein) from soon after hatch to approximately 100 days post hatch induced gonadal intersex (i.e. testis-ova) in males. Cheshenko *et al* (2008) explained that the general assumption is for phytoestrogens to inhibit steroidogenic enzymes by competitive inhibition with natural substrates for a particular enzyme. Also, some phytoestrogens are able to bind to respective receptors, and to directly interact with aromatase CYP19, which could lead to the inhibition of this enzyme, involved in synthesis of 17β-estradiol, thereby, affecting reproductive health of the fish. Both male and female vertebrates produce and use estrogens and androgens. A minor role for estrogens exists in males, such as in the regulation of GTH secretion by the pituitary gland. Estrogens levels in males may have more widespread effects, and may even play a role in fertility (Pait and Nelson, 2002).

The reported phytoestrogens identified in Pawpaw plant parts include β-sitosterol, saponins, and flavonoids (Krishna *et al*., 2008) and that of Moringa plant parts include flavonoids (quercetin and kaempferol), saponins, triterpenes, e.g. oleanolic acid-3-glucoside, and β-sitosterol (Anwar *et al*., 2007; Kumar *et al.,* 2010; Kumar *et al*., 2012). The two plants Pawpaw and Moringa abound in SSA and, that are used in traditional medicine. They have been demonstrated to possess abortifacient and/or antifertility properties (Das, 1980; Udoh and Kehinde, 1999; Bose, 2007). Thus, they can be exploited in the quest for a more reliable solution to tilapia precocious breeding, hence could encourage tilapia culture in rural SSA for poverty alleviation. This method of control if proven to be effective could be easier to adopt by poor fish farmers, particularly in SSA, since Pawpaw and Moringa seeds used in this study are available all year round in the tropics and subtropical regions.

Considering the problem associated with the use of androgenous hormonal treatment, for instance, environmental and public health concerns (Dabrowski *et al*., 2005) and the shortcomings of existing methods and techniques enumerated by Mair and Little (1991), an alternative approach is worth investigating. The study, therefore, aimed to investigate the potential of Pawpaw and Moringa seeds to suppress gonadal maturation of sexually immature Mozambique tilapia.

5.2 Material and Methods

5.2.1 Experimental Location and Facilities

The study was conducted in a recirculated aquaria system (RAS) based at the Welgevallen Experimental Farm, Stellenbosch, South Africa. Welgevallen Experimental Farm is located at 33°56' 33.95" S, and 18°51'56.15 "E. The experimental facilities consisted of a recirculating aquaria system that housed a total of 72 glass aquaria. The RAS consisted of two platforms; each platform had six racks, and each rack had two levels, with three tanks per level (Plate 3.1 Chapter 3). Each tank had a volume of 121L (dimensions 57cm x 53cm x 40cm). A detailed description of the RAS operation is given in Chapter 3.

5.2.2 Experimental Layout

The experimental design was a 3X5 factorial, with the three treatments being the commercially formulated tilapia feed as control or basal diet (BD), and the remaining two treatments of Pawpaw seed meal and Moringa seed meal. The experimental compounds were included at five different inclusion levels, i.e. 0, 0.5, 1.0, 2.0, 5.0, and 10.0 g/kg BD. The experimental diets were fed for a period of 60 days, after an acclimatization period of 7 days, to sexually immature Mozambique tilapia, weighing between 2 and 8g. At the end of the trial, 4 fish were sacrificed from each treatment for histological analysis of gonad integrity. The study was conducted between April 2011 and June 2011, and the experiment was repeated 5 times.

5.2.2 Experimental Animals and Compounds

A total of 1650 sexually immature Mozambique tilapia (*Oreochromis mossambicus*) weighing between 2 and 8g, were obtained from the general stock maintained at the Welgevallen Experimental Farm. The fish were randomly allocated to 55 tanks, at a stocking density of 30 fish (with unknown sex) per tank. The fish were allowed to acclimatize for 7 days, and after this period, the treatment diets were fed for period of 60 days. The fish were fed *ad libitum* (Baker, 1984) at three intervals, i.e. 08:00-09:00, 12:00-13:00, and 16:00-17:00.

Each aquarium was cleaned daily throughout the duration of the study. The mechanical filter (a plastic basket filled with Japanese mat, aquastones and foam) was cleaned every second day by removing the packaging and washing off all the solid and trapped materials. The larger tank units were cleaned on a weekly basis, with 10% of the volume being replaced and 1 kg common salt added to the system.

Pawpaw (*Carica papaya* Linn.) and Moringa (*Moringa oleifera* Lam.) seeds were used as potential sources of phytochemicals, collected and processed into powder as described in Chapter 3. The powder form was then included as a supplement in a commercially available tilapia diet, Aquanutro (Nutroscience (Pty) Ltd, South Africa), which was used as the control or basal diet (Table . More detailed on description on the preparation and storage of the experimental diets, is given in Chapter 3.

The composition of the experimental diets for the experiment, during which the effect of the seed powder on the gonadal activity of sexually immature Mozambique tilapia (*Oreochromis mossambicus*) was investigated, is presented in Table 5.1.

Table 5.1 Inclusion levels of Pawpaw (*C. papaya*) and Moringa (*M. oleifera*) seed powder in the experimental diets fed during Experiment II.

Treatment	Designation	Inclusion level of experimental compounds (g/kg basal diet) and designation				
(iv) Basal diet (BD; Control)*	C $_{(I-V)}$	No dosage level for Control Diet				
(v) BD + Pawpaw seed powder	P $_{(I-V)}$	P(0.5)	P(1.0)	P(2.0)	P(5.0)	P10.0)
(vi) BD + Moringa seed powder	M $_{(I-V)}$	M(0.5)	M(1.0)	M(2.0)	M(5.0)	M(10.0)

* Basal Diet: Commercial Aquanutro Tilapia starter diet. Five repeats per treatment (I-V). Fish per tank = 30.

5.2.4 Data recorded

Data that were recorded at the beginning and end of the trial period included the total length (TL) of the fish, wet body weight (BW), body depth (D), and the presence or absence of gonads. The body weight of the fish was measured using an electronic weighing scale (Electronic Balance, UWE, HGS-300, Capacity: 300 x 0.01g, Serial #-T5912) to the nearest 0.01g. Both the total length (i.e. from the tip of snout to tail end) and depth (i.e. base of dorsal fin along the opercula line to the bottom of ventral section of the body) (Skelton, 2001), were measured to the nearest 0.01mm using a calibrated measuring board.

After the 60-day trial four fish were randomly removed from each tank, and sexed into males and females using external features (genital papilla, the anal and dorsal fins). Sex was confirmed through examination of the gonads obtained by dissection. Internal body organs i.e. the testes and ovaries were weighed using electronic weighing scale (Electronic Balance, UWE, HGS-300, capacity: 300 x 0.01g, Serial # P9440), to the nearest 0.01g and fixed in Bouin's solution for histological study

After taking body measurements of the fish, they were anaesthitized with clove oil and then dissected. Each specimen was dissected with the aid of a pair of small scissors inserted through the vent. Also a semi-circular cut was made laterally on the side of the specimens for better observation. In each fish the state of the gonad (testes and ovaries) was visually observed for any deformity. For instance, in an ovary with matured eggs, the colour, relative size and shape of both gonads, were considered. In a testis, for instance, the structure, shape and texture were examined for any constriction. Any form of deformity or unusual appearance (e.g. sizes of gonads) was noted and photographed.

All the tissues in Bouin's were processed in an automatic tissue processor (SHANDON ELLIOT, Duplex Processor) by immersion in a serial dilution of alcohol (i.e. 80%, 90% and 100%), chloroform and molten paraffin wax, for 17 hours in the laboratory. The tissues were then embedded in paraffin wax at 60 ^{o}C, in an embedding instrument (LEICA EG 1160).

Using a microtome (Model, Bright 5040), 5µm -8µm thickness sections were cut. All tissues were oriented on the wax block in such a way to produce transverse sections (TS). However, few tissues of the testis were cut in longitudinal sections due to the size of the organ. For each specimen at least 3 sections were placed on a

glass slide in warm water. All the slides were stained for 25 minutes using LEICA AUTO STAINER, XL, with Ehrlich's haematoxylin and counterstained with eosin. Cover slips were attached to the slides with DPX mounting solution. DPX mountant for histology is a mixture of distyrene, plasticizer, and xylene

At the end of the trial period, histology preparations were examined to assess the extent of gonad damage by using a numerical grading scale that was developed based on a set of diagnostic criteria depicting observations of the histopathology of fish gonads. The slides were examined using a light compound microscope (OLYMPUS CH-30). Using a computer programme NIS Elements BR 3.0 (Driver Nikon Ds-U1, version 5.547) the image was enlarged onto a monitor for analysis. Photomicrographs of images shown on the screen were taken with Nikkon Digital Sight, DS Fi1 camera mounted on Zeiss-OLYMPUS CH-30 microscope. On the NIS Elements BR 3.0 programme, the images were resized, sharpened and colour adjusted prior to photographing. The criteria for the grading were set to be flexible enough to accommodate the various tissue changes, as proposed by OECD (2009). A detailed description of the various characteristics and how each one was recorded, is given in Chapter 3 (Table 3.6 and Table 37).

5.2.5 Statistical Analysis

Means of total length, total body weight, depth were computed using Microsoft Excel 2010. A one-way analysis (ANOVA) was performed to test for the significance of variance for the weight, length and depth traits between different treatments using SAS Version 9.1. The GSI values were subjected to logarithmic transformation to stabilise the variance to obtain a normal distribution. The correlation between the inclusion levels and gonad development (i.e. GSI) was estimated by means of a one-way ANOVA, using SAS (Version 9.1), and a pair-wise t-test of the Least Square Means (LSM) at a significance level of 95% ($P<0.05$). Due to the small sample size (n=4) per treatment all the fish (both male and female) were pooled, and analysed to assess the overall effect of Pawpaw and Moringa as EDCs. The frequency of gonad damage and /or integrity was analysed by using a t-test.

5.3 Results

5.3.1 Biological: Body and Organ Measurements

The morphometric parameters (i.e. total length, total wet body weight, body depth, and gonadosomatic index), recorded for both male and female tilapia are presented in Table 5.2 and Table 5.3.

In the fish which were identified as males, fish fed Pawpaw, the largest mean length was observed in P1.0g/kgBD, 128.06 ± 3.60, and weight was 39.12 ± 2.08, in P2.0g/kgBD. In the Moringa fed fish, the largest mean length was observed in M10.0g/kgBD,135.65 ± 3.54, and the mean weight was 48.30 ± 3.64, in M10.0g/kgBD.

In the fish identified as females, fish fed Pawpaw meal, the largest mean length was observed in P0.5g/kgBD, 127.00 ± 2.87, and weight was 42.56 ± 3.28, in P0.5.0g/kgBD. In the Moringa fed fish, the

largest mean length was observed in M2.0g/kgBD,125.17 ± 4.38, and the mean weight was 37.47 ± 3.39, in M2.0g/kgBD.

Table 5.2 Morphometric parameters (mean ± SE) of sexually immature Mozambique tilapia (*Oreochromis mossambicus*) males that received a diet containing different levels of Pawpaw and Moringa Seed meal as part of their basal diet (i.e. 0, 0.5, 1.0, 2.0, 5.0, 10.0 g/kg basal diet) over a period of 60 days.

Treatment	N	Body length (mm)	Body weight (wet; g)	Body depth (mm)	Gonadosomatic Index (%)
Control (C)	16	$127.75^{a} \pm 3.34$	$39.06^{a} \pm 2.80$	$36.00^{a} \pm 1.10$	$0.27^{a} \pm 0.18$
Pawpaw					
P0.5	15	$127.73^{a} \pm 3.22$	$40.11^{a} \pm 2.58$	$37.80^{a} \pm 1.33$	$0.32^{a} \pm 0.18$
P1.0	16	$128.06^{a} \pm 3.60$	$40.72^{a} \pm 2.70$	$37.13^{a} \pm 1.27$	$0.33^{a} \pm 0.11$
P2.0	13	$124.92^{a} \pm 2.12$	$39.12^{a} \pm 2.08$	$37.62^{a} \pm 0.95$	$0.20^{b} \pm 0.06$
P5.0	15	$127.53^{a} \pm 3.32$	$43.77^{a} \pm 4.19$	$39.27^{b} \pm 1.86$	$0.24^{a} \pm 0.09$
P10.0	12	$125.83^{a} \pm 3.23$	$41.09^{a} \pm 3.22$	$38.42^{b} \pm 1.67$	$0.23^{a} \pm 0.07$
Moringa					
M0.5	12	$124.25^{a} \pm 2.67$	$38.26^{a} \pm 2.28$	$36.92^{a} \pm 1.50$	$0.30^{a} \pm 0.06$
M1.0	14	$126.93^{a} \pm 3.84$	$41.83^{a} \pm 3.84$	$38.07^{a} \pm 1.45$	$0.17^{b} \pm 0.05$
M2.0	14	$125.79^{a} \pm 3.90$	$40.40^{a} \pm 3.76$	$39.50^{b} \pm 1.86$	$0.37^{a} \pm 0.17$
M5.0	11	$130.27^{a} \pm 2.44$	$43.37^{a} \pm 2.08$	$36.09^{a} \pm 0.64$	$0.31^{a} \pm 0.07$
M10.0	17	$135.65^{b} \pm 3.54$	$48.30^{b} \pm 3.64$	$38.71^{b} \pm 1.46$	$0.23^{b} \pm 0.09$

[a, b] Different superscripts in a column denote significant differences ($P<0.05$)

Table 5.3 Morphometric parameters (mean ±SE) of sexually immature Mozambique tilapia (*Oreochromis mossambicus*) females that received a diet containing different levels of Pawpaw and Moringa Seed meal as part of their basal diet (i.e. 0, 0.5, 1.0, 2.0, 5.0, 0.0 g/kg basal diet) over a period of 60 days.

Treatment	N	Body length (mm)	Body weight (wet; g)	Body depth (mm)	Gonadosomatic index (%)
Control (C)	4	$118.50^{a} \pm 3.43$	$30.04^{a} \pm 2.69$	$33.00^{a} \pm 4.12$	$2.15^{a} \pm 0.53$
Pawpaw)					
P0.5	5	$127.00^{b} \pm 2.87$	$42.56^{b} \pm 3.23$	$38.40^{b} \pm 2.42$	$3.99^{b} \pm 1.78$
P1.0	4	$126.25^{b} \pm 2.44$	$38.08^{b} \pm 2.14$	$35.50^{a} \pm 1.50$	$2.38^{a} \pm 0.48$
P2.0	7	$126.57^{b} \pm 3.62$	$40.01^{b} \pm 3.75$	$36.14^{a} \pm 3.18$	$2.37^{a} \pm 0.87$
P5.0	5	$122.80^{a} \pm 3.57$	$38.77^{b} \pm 3.74$	$36.80^{a} \pm 2.55$	$2.23^{a} \pm 0.56$
P10.0	8	$119.13^{a} \pm 3.43$	$35.24^{a} \pm 2.56$	$35.63^{a} \pm 1.14$	$2.95^{b} \pm 1.15$
Moringa					
M0.5	8	$117.88^{a} \pm 2.12$	$33.91^{a} \pm 1.72$	$35.00^{a} \pm 1.50$	$3.81^{b} \pm 1.29$
M1.0	6	$118.17^{a} \pm 2.56$	$35.13^{a} \pm 2.20$	$34.11^{a} \pm 2.38$	$7.45^{b} \pm 2.57$
M2.0	6	$125.17^{a} \pm 4.38$	$37.47^{a} \pm 3.39$	$36.33^{a} \pm 1.11$	$2.15^{a} \pm 0.58$
M5.0	9	$121.00^{a} \pm 2.20$	$34.13^{a} \pm 2.43$	$33.00^{a} \pm 2.43$	$2.87^{b} \pm 1.66$
M10.0	3	$123.67^{a} \pm 1.97$	$36.91^{a} \pm 1.61$	$31.67^{a} \pm 2.14$	$1.32^{a} \pm 0.39$

$^{a, b}$ Different superscripts in a column denote significant differences ($P<0.05$)

The morphometric parameters (i.e. total length, TL; total wet body weight, BW; body depth, D, and gonadosomatic index, GIS) recorded for both male and female fish in the study presented in Table 5.2 and Table 5.3 indicate that the longest TL (135.65±3.54 mm; $P<0.05$), heaviest BW (48.30±33.64g; $P<0.05$) and highest value for D (38.71±1.46mm; $P<0.05$), as well as the largest number of male fish were recorded for the group receiving 10.0g MSM/kg BD (MSM10; $P<0.05$), when compared to the control and other treatment groups.

5.3.2 Influence of Pawpaw and Moringa on gonad development

Table 5.4 presents the resulting degree of gonad differentiation observed and recorded for sexually immature Mozambique tilapia that received various treatment diets. Plate 5.1 and Plate 5.2 represent a comparison of normal and abnormal gonad differentiation observed in sexually immature Mozambique tilapia that received various treatment diets over a period of 60 days.

Table 5.4 The effect of Pawpaw seed meal (P) or Moringa seed meal (M) on gonad differentiation of sexually immature Mozambique tilapia (*Oreochromis mossambicus*; 2-8g) that received diets containing different levels (0; 0.5; 1.0; 2.0; 5.0; 10.0g/kg control diet) of P and M include as supplements to a commercial tilapia diet (control) for a period of 60 days.

Treatment	N	Observed degree of gonadal damage per gender (%)							
		No effect (normal)		Minimal effect		Moderate effect		Pronounced effect	
		♂	♀	♂	♀	♂	♀	♂	♀
Control diet (BD)	20	10^{c}	4^{c}	5^{c}	1^{c}	-	-	-	-
Pawpaw									
P 0.5	20	7^{c}	4	3^{c}	-	6^{c}	-	-	-
P1.0	20	2^{c}	3^{c}	5^{c}	2^{c}	8^{c}	-	-	-
P2.0	20	1^{c}	5^{c}	7^{c}	1^{c}	5^{c}	-	1^{b}	-
P5.0	20	-	2^{c}	4^{c}	2^{c}	40^{a}	10^{b}	15^{a}	-
P10.0	20	-	10^{a}	15^{a}	10^{a}	25^{a}	10^{a}	30^{a}*	-
Moringa									
M0.5	20	15^{c}	15^{c}	5^{c}	25^{c}	25^{c}	-	15^{c}	-
M1.0	20	10^{b}	5^{b}	20^{b}	20^{b}	20^{b}	5^{b}	20^{b}	-
M2.0	20	5^{b}	10^{b}	5^{c}	30^{b}	30^{b}	-	20^{b}	-
M5.0	20	-	5^{b}	10^{b}	35^{b}	20^{b}	5^{b}	25^{b} *	-
M10.0	20	-	5^{b}	20^{a}	5^{c}	45^{a}*	-	25^{a}*	-

*:Ova-testes observed in male gonads; columns with same superscript are statistically not different $p<0.001$)

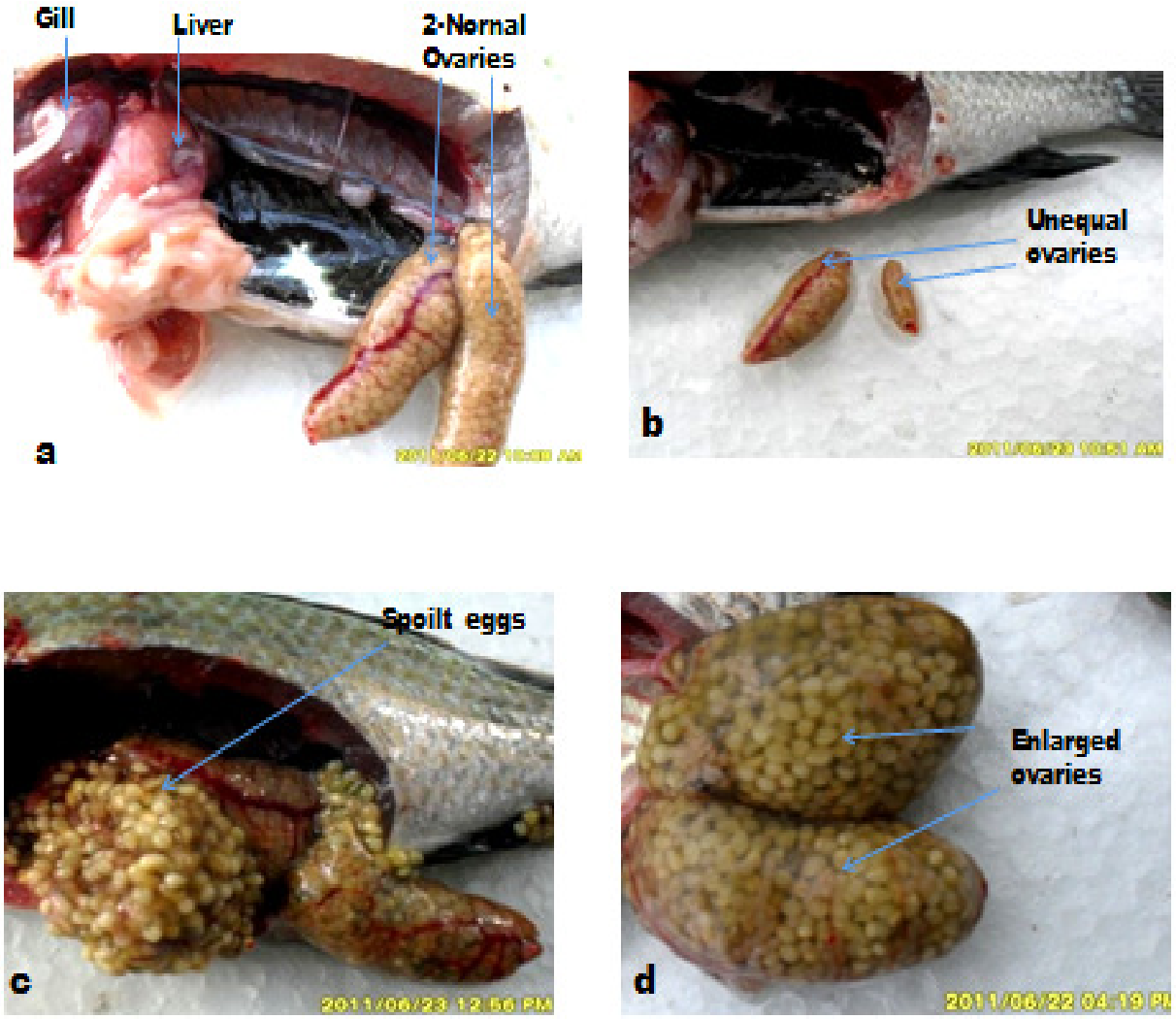
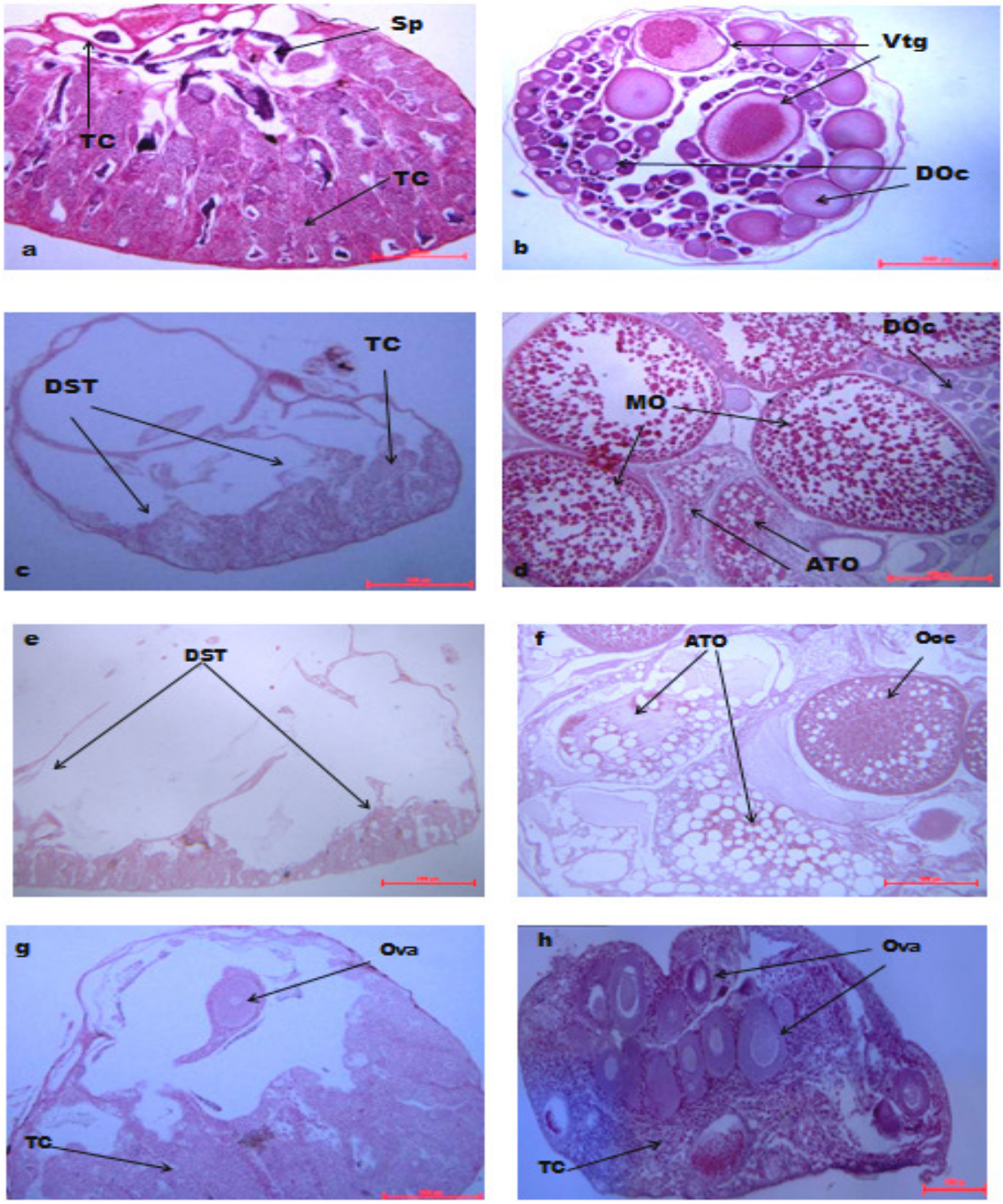


Plate 5.1 A comparison of the gonad differentiation of sexually immature Mozambique tilapia (*Oreochromis mossambicus)* that received diets containing different levels (0, 0.5, 1.0, 2.0, 5.0, & 10.0g/kg basal diet) of Pawpaw seed meal (P) or Moringa seed meal (M) as supplements to a commercial tilapia diet, over a period of 60 days. a) Normal ovaries, similar in size and shape, b) unequal sized gonads, c) enlarged ovaries with degenerated or deformed eggs (abnormal colour), and d) enlarged ovaries.



Plates 5.2 Gonad differentiation observed in sexually immature Mozambique tilapia (*Oreochromis mossambicus*, 2-8g) that received diets containing different levels (0, 0.5, 1.0, 2.0, 5.0, & 10.0g/kg basal diet) of Pawpaw seed meal or Moringa seed meal that were included as supplements in a commercial tilapia diet over a period of 60 days (X500 magnification). a) Normal developing testis (0g/kg BD); b) Normal developing ovary (0g/kg BD); c) Testis observed in fish receiving 2.0g PSM/kg BD; d) Ovary observed in fish receiving 2.0g PSM/kg BD; e) Testis observed in fish receiving 5.0g PSM/kg BD; f) Ovary observed in fish receiving 5.0g PSM/kg BD; g) Testis-ovarian tissue observed in fish receiving 10.0g PSM/kg BD; and h) Testis-ovarian tissue observed in fish receiving 10.0g MSM/kg BD. ATO-atretic oocyte; Doc-developing oocytes; DST-degenerating seminiferous tubules; MO-maturing oocytes; Sp-spermatids; TC-testicular cells (i.e. sperm germ cells); Vtg-vitellogenic oocytes. Bar = 500µm.

5.4 Discussion

5.4.1 Biological parameters, Organ Measurements and Gonadal Defects

The longest TL (135.65±3.54 mm; P<0.05), heaviest BW (48.30±33.64g; P<0.05) and highest value for D (38.71±1.46mm; P<0.05), as well as the largest number of male fish were recorded for the group receiving 10.0g MSM/kg BD (MSM10; P<0.05), when compared to the control and other treatment groups.Despite these significant differences, no reproductive activities were observed throughout the experimental period of 60 days when the fish received the respective treatment diets. However, during the last week of the trial, a female from the Control group (CG) was observed brooding eggs in her mouth. This observation means that the Pawpaw and Moringa seeds rendered the treated fish incapable of successful reproduction within the 60 days. It affected the gonadal maturation, thereby interferring in spawning. Another possible explanation is that the cocktail of phytoestrogenic compounds could interfere with the maturation of gametes that resulted in the asymmetrical development and morphological deformation of the gonads, retardation of sexual maturity, change in the duration of the periods of gonadal development, and increase in the number of fishes omitting spawning (Pavlov *et al.,* 2009), and hence the delayed reproduction.

When the sexual differentiation of the fish is considered, feeding diets containing P or M resulted in 70% males (155/220) being identified, compared to only 30% females (65/220). The highest GSI was assigned to males from the group that received 2.0g M/kg BD (0.37 ± 0.17; M2.0), and the highest GSI was assigned to females from the group that received 1.0g Moringa/kg BD (7.45 ± 2.57, P; M1.0) (Table 5.1). The lowest GSI was assigned to females from the group found in treatment group M1.0g/kg, with the lowest 1.3200±0.394 in M10.0g/kg, which was not significant at (p<0.05) from the control 2.1525±0.534. The assignment of the GSI allows one to quantify changes that take place in the gonad, i.e. in terms of differentiation and function. A higher GSI value implies that an animal is ready to spawn. In the wild, species that reproduces seasonally, the GSI gradually increases and reaches a maximum as soon as the environmental conditions are favourable for the fish to spawn. For species that have a long duration of spawning or those that are batch spawners, the GSI does not involve such a dramatic difference in size (Pavlov *et al.,* 2009), as was observed in this study.

According to Mañanos *et al.* (2009), under optimal conditions the sensory and endocrine systems of fish are able to recognize the possibility of a high survival rate of the offspring, resulting in the parent fish to allocate or invest more energy into reproduction and spawning. *Oreochromis* species are considered batch spawners, and exhibit a regular periodicity in gonadal growth throughout a breeding season, with females ovulating more than once per season. Tyler and Sumpter, (1996) reported that the GSI of *Oreochromis* species rarely exceeds 10% of body weight, but fluctuates between 2 and 10, between spawning seasons. The GSI observed in this study also fluctuated between 2 and 10%, in agreement with Tyler and Sumpter (1996). The GSI scores assigned in this study indicate that there is a progressive increase in gonad size in both the males and females implying that the environmental conditions within the experimental unit were conducive for sexual development of the fish. However, no reproduction occurred throughout the entire 60-day period.

The gonads of a sexually maturing Mozambique tilapia (*Oreochromis mossambicus*) resembles that of a gonochoristict teleost fish, where the female gonad consist of two distinct ovaries of similar size, which are joined at their posterior ends, and open through a common oviduct in the urogenital papilla. In male tilapia, the paired testes are located in the dorsal body wall and joined at the urogenital opening (Yamazaki, 1983; Morrison *et al*., 2006). In this study, normal ovaries were observed in fish receiving the Control diet (Plate 5.1a). When the gonadal development of fish sampled from the respective treatment groups are compared to that of the CG, deformed ovaries were observed in fish fed with diets containing 2.0, 5.0 and 10.0g/kg basal diet of P or M (Plate 5.1 a-d). The most prominent deformities included ovaries that were unequal size (Plate 5.1b), enlarged ovaries (Plate 5.1c & d), and enlarged ovaries filled with a large number of white to cream-coloured eggs (Plate 5.1 c). Other visible deformities included ovaries twisted at the mid-section, a single ovary or fused ovaries, flap testis, a testis strand with dotted ova and testes with constrictions in the mid- and posterior regions. The majority of the females who carried deformed ovaries, had distended abdomens. With The eggs in the ovarian cavity will degenerate and reabsorbed, in such situations could cause the death of the fish (Mañanos *et al.,* 2009).

The appearance of eggs observed in females from the CG (Plate 5.1a) conformed to what has been described for the *Oreochromis* species, i.e. as having an oval shape, being relatively large, and being yolky with yellowish to ochre colouration or orange-yellow in colour (Rana, 1988; MacIntosh and Little, 1995; Morrison *et al*., 2006). *Oreochromis* species cultured in aquaculture production systems are reported to normally produce eggs that are ochre (i.e. red to yellow earth) in colour. The intensity of colour, however, is influenced largely by nutrition, for example, the quantity of pigments such as carotenoids in the diet of brood fish (Rana, 1988). *O. mossambicus* eggs produces eggs that are yellow in colour (Shelton and Popma, 2006). When the ova of fish in this study are considered, they contained eggs that were white to cream in colour (Plate 5.1b-d). This is potentially an indication that the eggs were abnormally formed. This is supported by the fact that although eggs were observed in the ovaries of the fish that were samples in this study, no spawning and/or females brooding eggs were observed for the treatment groups receiving 2.0, 5.0 and 10.0 g/kg basal diet of both P and M.

Oocyte enlargement can be ascribed to the accumulation of yolk, formed from vitellogenin (a female specific protein), which is synthesized by liver in response to 17β-estradiol, released into the blood stream, and later transported to the ovary (Nagahama, 1983; Tyler and Sumpter, 1996). The phytochemicals present in the Pawpaw and Moringa potentially mimicked the biological effect of endogenous hormones such as 17β-estradiol, preventing the processing and incorporaton of vitellogen, resulting in liquefaction or vacuoles forming in the eggs, reducing the viability of the eggs.

Atresia a process of follicle degeneration, or the resorption of developing oocytes could occur if estrogens which are indispensable for the growth and maturation of follicles activities are curtailed. Atretic follicles exhibit decreased estrogen production and a lower estrogen/androgen ratio in the follicular fluid, suggesting the importance of local estrogens for the maintenance of healthy follicles (Mañanos *et al.,* 2009). Atretic follicles are also characterised by reduced aromatase activity, leading to decreases of estrogen production

and accumulation of androgen (Blazer, 2002; Mañanós *et al*, 2009). The observed enlarged gonads could occur possibly due to the phytoestrogens from the Pawpaw and Moringa antagonizing the effects of estrogens by binding to the receptor but not activate and / or alter the pattern of synthesis and metabolism of it. In this case, the presence of the chemical on the receptor prevented the binding of the natural hormone preventing the final maturation and ovulation. Thus, instead of the fish ovulating and spawning the eggs stays in the ovary leading to its enlargement. From the above explanation it is clear that the enlarged ovaries could not have been follicular atresia, but different process did occurred to elicit the observed effect in the fish.

The female fish in captivity experiences reproductive dysfunction, caused by stress and absence of environmental cues (i.e. light and temperature) for reproduction. Studies have shown that three forms of reproductive dysfunction that could affect egg quality involves inhibition of vitellogenesis, inhibition of the final oocyte maturation and inhibition of the process of spawning (Mañanós *et al*, 2009). According to Pavlov *et al.* (2009), the quality of an egg depends on its inherent properties and the environment in which the egg develops from fertilization. The egg's ability to produce a viable offspring depends on the genotype, as well as the morphological, biochemical and physiological processes happening inside the egg. Furthermore, poor egg quality (i.e. spoilt eggs) can be caused by the dysynchoinisation in the processes of egg maturation and ovulation which could bring about under-ripening or over-ripening and disturbances in egg maturation (Mañanós *et al*, 2009; Pavlov *et al*, 2009). Therefore, the spoilt ova which were encountered, might have been caused by dysynchronization of egg maturation.

It has been established that, in teleost fish at the end of gametogenesis pituitary Luteinizing-Hormone (LH) secretion induces the synthesis of maturation-inducing steroids (MIS), which regulate the process of gonadal maturation. After maturation is completed, a Gonadotropin Releasing Hormone (GnRH) induced LH surge stimulates ovulation. Some of the reported anomalies attributed to endocrine disrupting compounds in aquatic environments include morphological deformation of the gonads and asymmetrical development. One major reproductive dysfunction of females is inhibition of spawning which prevents the eggs from shedding out of the oviduct thereby the ovulated eggs remain in the ovarian cavity (Mañanós *et al*, 2009; Pavlov *et al,* 2009). Therefore, the deformed gonads and spoilt eggs in this study were as a results of the presence of the phytoestrogens present in the Pawpaw and Moringa seeds. This could account for the enlargement of the ovary with a huge volume of over-ripening eggs (Plates 5.1c & d). This clearly indicates that Pawpaw and Moringa possess compound or chemicals that affect the integrity of gonads and eggs; therefore, they can be used to manipulate reproduction in developing or maturing tilapia to prevent it spawning in aquacultural system.

5.4.2 Histological assessment of gonadal damage severity

The results from the gonadal damage severity shown in Table 5.3 and Figures 5.2 and 5.3 indicate that quite a number (n=30) of gonads were severely damaged. Pronounced severity on integrity of the gonad was observed in P5.0, P10.0 and M10.0g/kg basal diet, but no significant differences occurred at $P<0.05$, but

significant compared to the lower inclusion levels (0, 0.5, 1.0 and 2.0 g/kg basal diet) at $P<0.05$ ($P = 0.0366$). A testis with egg producing cell was found in the male gonads of tilapia fed with 10.0g/kg basal diet of Pawpaw or Moringa seed powder, called testes-ova (Jobling *et al,* 1998; OECD, 2009). The severity of the gonadal damage is based on a set of criteria that describe the state of the gonad (see Chapter 3)

Endogenous sex steroids have been identified to influence two broad categories of physiological systems in teleost fish, namely organizational and activation (Arnold and Breedlove, 1985; Hiramatsu *et al*., 2005). In most investigations the three main effects that have been identified as evidence of EDC effects on the apparent reproductive endocrine disruption in aquatic organisms are: (i) intersex fish (male and female gonadal characteristics in gonochoristic or normally separate sex fish), or the testis-ova (Jobling *et al.,* 1998), (ii) elevated levels of a female egg protein in male and immature or juvenile fish (Janssen *et al*., 1997; Lye *et al*., 1998), and (iii) degeneration of gonadal tissue (Janssen *et al*., 1997; Lye *et al*., 1998). Two of these 3 main effects were identified in this study, namely the presence of testis-ova and degeneration of the gonadal tissues, established from numerous studies as the most widely used indicators of exposure to estrogenic, anti-estrogenic, or anti-androgenic endocrine-disrupting chemicals (Jobling *et al*., 1998; Tremblay and van der Kraak, 1999; Pait and Nelson, 2002; Manning, 2005; Mills and Chichester, 2005). The testis-ova condition is caused by exposure of the fish to estrogenic compounds during critical stages of gonadogenesis (Balzer, 2002), and it is one of the critical conditions identified in this study. The severity on integrity of the gonad observed in P5.0, P10.0 and M10.0 (Figures 5.2 and Plate 5.2), included the presence of testis-ova and degeneration of the germ cells, conform to the above described for exposure to EDC. This could mean that Pawpaw and Moringa seed possess phytoestrogenic compounds. This result from this study clearly indicates that Pawpaw and Moringa seeds contain phytoestrogens, with the capacity to induce the observed structural changes.

The general held view on the mechanism by which EDCs affect endogenous hormones is to mimic or antagonize the effects of endogenous hormones. Studies over time have established that EDC exert their effects through a number of different modes of action or mechanisms on aquatic organisms, as enumerated by Pait and Nelson (2002). Both Pawpaw and Moringa seed contais a cocktail of phytoestrogens, therefore their mode of action could be a combination of two or more of any of these five mechanisms of interfering with the activity of endogenous hormones. The mechanisms (i) simulating the effects of biological activity of endogenous hormones, (i.e. estrogens and androgens), binding to a cellular receptor leading to an false response by initiating the cell's normal response to the naturally occurring hormone at the wrong time or to an excessive extent (i.e. agonistic effect) (ii) antagonizing the effects of endogenous hormones by binding to the receptor without activate it, blocking the binding of the natural hormone (i.e. antagonist effect); (iii) changing the pattern of synthesis and metabolism of normal hormones, i.e. they may interfere with the metabolic processes in the body, affecting the synthesis or breakdown rates of the natural hormones, (iv) modifying hormone receptor levels and, particularly in organs that are related to reproductive functions which are known to have high levels of receptors, their specificity and affinity, and (v) may also interfere with the binding proteins that act to transport endogenous hormones to their destination, here they may bind to transport proteins in the blood, thus altering the amounts of natural hormones that are present in the

circulation (Blazer, 2002; Pait and Nelson, 2002; Manning, 2005; Mills and Chichester, 2005; Evans *et al*, 2011). Although hormones reach all parts of the organism's (e.g. fish) body, only target cells with compatible receptors are able to respond. Binding of the receptor and a hormone could alter the cell's existing proteins or activate genes that will build a new protein (Moutsatsou, 2007). Results indicate that exposure of an organism to levels of EDCs or natural hormones which can interfere with the proper functioning of the endocrine system could have severe effect on the reproductive endocrine function leading to alteration in reproductive development (Jobling *et al*, 1998; Damstra *et al*, 2002; Manning, 2005; Mills and Chichester, 2005; Cheshenko *et a*l, 2008; Sassi-Messai *et al;* 2009; Blazer *et al*, 2012). The structural changes as seen in Plate 5.2 shows that Pawpaw or Moringa interacted with the endogenous hormones to elicit the observed effects of *O. mossambicus* gonad integrity.

5.4.3 Management of reproduction in tilapia-Puberty control

The wild Mozambique tilapia (*Oreochromis mossambicus*) reaches maturity at a size that ranges from 110mm to 285mm. In a fast growing culture environment *O. mossambicus* could spawn at an age of three months, weighing between 60 and 100g, but in a poorly fertilised pond fish might weight as low as 15g (Shelton and Popma, 2006).

Puberty is "the developmental period comprising the transition from an immature juvenile to a mature adult state of the reproductive system, i.e. the stage of development during which an individual becomes capable of reproducing sexually, implying functional competence of the brain–pituitary–gonad (BPG) axis" (Okuzawa, 2002). During puberty the two main functions of the gonad become active, namely, the production of germ cells and the synthesis of reproductive (steroid) hormones. These hormones regulate germ cell development in both females (e.g. oestrogens and vitellogenesis) and males (e.g. androgens and spermatogenesis). Puberty is considered completed with the first batch of fertile gametes (Okuzawa, 2002; Munakata and Kobayashi, 2010; Taranger *et al*., 2010)

In the natural environment the effect of EDCs would depend on various factors including: the potency or efficacy of the EDC; its concentration; duration of exposure; bioconcentration potential; presence of other EDCs; life stage; season; other environmental stressors present (e.g., temperature, photoperiod, salinity, and other contaminants) and mobility of the individual (Pait and Nelson, 2002). In aquarium setting, the space available for the fish appears to be in more proximity to the fish in comparison to the wild. The phytoestrogen provided or incorporated as part of Pawpaw and Moringa seed meal, can be manipulated in such a way that the feed can fit or depict the criteria described by Pait and Nelson (2002).

The endogenous hormone, estrogen that controls sexual differentiation, maturation and reproduction (Jobling *et al*, 1998), is biosynthesised through the steroidogenic enzyme cytochrome P450 aromatase, which converts androgens to oestrogens (Afonso *et al*., 2001). So any compound or chemical that has the capacity to replace an estrogen in its activity in the cell, will eventually affect the functional activity exerted by

the estrogen. Generally, the assumption is that phytoestrogens inhibit steroidogenic enzymes by competitive inhibition with natural substrates for a particular enzyme.

In fish, the enzyme aromatase CYP19 is often the target of EDCs, which modifies its expression and function, changing the level of estrogen production, disrupting estrogen biological processes including malfunctioning of the reproductive system (Cheshenko *et al*, 2008; Zhao and Mu, 2011). And phytoestrogens, such as daidzein, genistein, quercetin, kaempferol, β-sitosterols saponins and the triterpenes which appear to be potent modulators of the activity of the aromatase enzyme and reported to exist in Pawpaw and Moringa, can be used to manipulate puberty in tilapia to control the precocious breeding behaviour in production systems.

According to Zhao and Mu (2011) the regulation of reproduction by the brain is through the brain-pituitary-gonad (BPG) axis, which controls the pituitary gonadotropin hormone including the gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH) and luteinizing hormone (LH). The FSH and LH are the hormones which induce the synthesis of the sex hormones such as 17β-estradiol (E2) and testosterone (T), which control ovulation and sper development. The E2 operates on a positive and negative feedback roles on the gonadotropin or its upstream hormone synthesis and secretion, through direct interaction with estrogen receptors. In this way phytoestrogen plays a role in mimicking or antagonizing the E2 function. It is believed that the BPG aixs is activated at the onset of puberty, which is also depended on several external and internal factors such as photoperiod, water temperature, somatic growth and gonadal status (Okuzawa, 2002; Taranger *et al*, 2010). For instance, photoperiod has been manipulated to delay the initiation of puberty in salmonids (Taranger *et al*, 2010). Both endogenous and exogenous steroid hormones are considered to have positive effect on pubertal development (Schulz and Goos, 1999. It has been proposed that sex steroids are the natural inducers of onset of puberty in male African catfish, and that a long-term treatment of 11ketotestosterone or its precursor stimulated testicular growth and spermatogenesis, probably through direct effect on the testes (Cavaco *et al*, 1998; Schulz and Goos, 1999).

The Pawpaw and Moringa seed powder used in this experiment have been found to contain some phytoestrogens β-Sitosterol; genistein, diadzein, equol, quercetin and kaempferol (Moutsatsou, 2007; Zhao and Mu, 2011). These phytoestrogens that have the ability to interfere with the activity of BPG axis (known to control puberty and reproduction) could affect puberty in fish. Judging by the disturbances as observed in the gonads of fish fed meals containing P5.0, P10.0 and M10.0g/kg BD (Figures 5.2 and 5.3), Pawpaw and Moringa have the capacity to effect changes in the reproductive system of fish. The possible ways by which Pawpaw and Moringa which contains multiple forms of phytochemicals with proven estrogenic or androgenic activity can elicit the effects shown above could be through two or more routes described above by (Blazer, 2002; Pait and Nelson, 2002; Manning, 2005; Mills and Chichester, 2005; Evans *et al*, 2011).

5.5 Conclusions

It can be concluded that Pawpaw and Moringa Seed powder have the capacity to delay puberty, and restrain the precocious breeding in tilapias, thus could be used successfully to control reproduction in tilapia. Further studies need to be conducted on the appropriate inclusion levels, duration of application and size of the fish for commercial or practical application.

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Chapter 6

The Effect of Pawpaw Seed meal and Moringa Seed Meal on The Sexual Differentiation of Mozambique Tilapia Fry (*Oreochromis mossambicus*)

Abstract

The use of all-male populations is the prefered option to control reproduction in tilapia aquaculture and to utilise the superior growth characteristics of males compared to females. The most commonly used method to produce all-male tilapia populations is the use of the synthetic steroid, 17α-methyltestosterone (MT) to induce sex-reversal. However due to the perceived health and environmental hazards, the use of natural reproductive inhibitors should be explored for use in aquaculture. Phytoestrogens are phytochemicals that induce biological responses in vertebrates and mimic or modulate the actions of endogenous oestrogens (such as 17β-estradiol), usually by binding to estrogen receptors. This study evaluated the effect of Papaw or Moringa seed as potential endocrine modulators that could affect sexual differentiation in juvenile Mozambique tilapia, *Oreochromis mossambicus*. Experimental treatments were respectively Pawpaw seed powder (P) during the 1st and Moringa seed powder (M) during the 2nd trial, into a commercial tilapia starter feed (15g/kg Basal Diet), together with a Negative Control (NC) comprising of the Basal Diet, and Positive Control containing 17α-Methyltestosterone (MT), fed to first exogenous feeding *O. mossambicus* fry for a period of 30 days. The sex ratio and survival rates were recorded at age 90 days. Phenotypic sex was determined by macro-examination of gonads. Results of the 1st trial indicate that Pawpaw Seed (P) had a significant effect on the sexual differentiation of *O mossambicus* fry, whilst neither the Positive Control (MT) nor the Negative Control (NC) induced any significant deviation from the expected 1:1 ratio. In the 2nd trial both the Moringa Seed and the Positive Control treatments showed a significant effect on the sex ratio of tilapia fry. None of the treatments in any of the trials had any significant effect on survival rate. The inclusion of Pawpaw and Moringa seed in the diets of first exogenous feeding of mixed sex *O. mossambicus* fry showed some phytoestrogenic effects on the gonadal development without any effect on survival.

6.1 Introduction

Tilapia have become one of the most important food fishes of the world with a significant increase in production from 383,654 metric tonnes (mt) in 1990 to 3,497,391 mt in 2010 (FAO, 2012). Much of the growth can be ascribed to the increased efficiency of monosex (i.e. all-male) populations obtained through hormonal manipulation of early sexual differentiation of fry (El-Sayed *et al.,* 2012). In Sub-Saharan Africa (SSA), tilapia culture is still hampered by uncontrolled reproduction in ponds, which result in the harvest of stunted fish with low nutritional and commercial value. Most of the advanced biotechnologies developed to manage or manipulate the precocious breeding strategies of tilapia in culture systems are to a large extent not accessible to farmers in rural and peri-urban SSA. Studies over the last two decades have indicated only monosex tilapia production systems in SSA to be financially viable in terms of commercial small-scale culture, together with polyculture systems of the African catfish (*Clarias* species) in Ghana and Nigeria (Stadtlander *et al.,* 2008).

The critical developmental period of differentiation of fish sex into male or female is plastic and is controlled by genetic, physiological and/or environmental factors (Devlin and Nagahama, 2002; Rougeot *et al.,* 2008). Sexual differentiation can therefore be manipulated towards the preferred gender, such as all-male in the

case of tilapias (Pandian and Varadaraj, 1990; Popma and Lovshin, 1995; Phelps, 2006; El-Sayed, 2006) that contribute significantly towards the expansion of tilapia culture.

Among the four major ways of producing all-male tilapia, i.e. manual/hand sorting, hybridization, genetic manipulation, and hormonal sex reversal, the technique of using androgenic hormones to sexually reverse to an all-male stock has become the most common practice in commercial culture. In spite of its laborious nature manual sorting remains the most common practice in rural SSA culture. Hybridization and genetic manipulation remain unavailable to SSA farmers due to technical constraints and cost.

The use of hormones for sex reversal is however, under increasing public scrutiny due to perceived potential health risks, environmental impacts, and social constraints (El-Sayed, 2006; Lückstadt *et al.,* 2006). In most developing countries, including SSA there is no effective regulation on the use of androgenic hormones while it also remains difficult to acquire due to cumbersome import and export regulations.

Considering the problems associated with the use of androgenous hormonal treatment, the shortcomings of the existing methods and techniques enumerated by Mair and Little (1991) and the unavailability to rural and peri-urban SSA, an alternative approach is worth investigating.

Phytoestrogens as Endocrine Disrupting Chemicals

Natural reproduction inhibitors found in medicinal plants provide an alternative for the control of sexual differentiation in fish. Studies have shown that some phytochemicals are natural steroid-like substances with estrogen-like biological activity, called phytoestrogens (i.e. estrogenic compounds found in plants). A phytoestrogen may be defined "as any substance or metabolite that induces biological responses in vertebrates and mimic or modulate the actions of endogenous oestrogens (such as 17β-estradiol), usually by binding to estrogen receptors" (Patisaul and Jefferson, 2010).

Medicinal plants have been used successfully to induce sterility in laboratory animals (Bodharkar *et al.*, 1974; Das, 1980). It raises the question whether such plant bioactive chemicals which act as the natural reproductive inhibitory agents could be used to control tilapia recruitment in ponds. The effect of phytoestrogens on fish was first reported in trout and sturgeon (Pelissero *et al.*, 1991; Pelissero and Sumpter, 1992; Pelissero *et al.*, 1996). Estrogens are steroid hormones involved in the control of important reproduction- related processes, including sexual differentiation, maturation and exhibition of secondary sex characteristics, which indicates that a proper function of the fish body requires a balance of estrogens. Nakamura *et al.* (1998) emphasized that, in the teleost fish ovarian differentiation begins at an earlier stage than testicular differentiation, with biosynthesis of estradiol -17β from cholesterol, through enzymatic activity of aromatase. In the biosynthesis of estrogen, at the final step cytochrome P450 aromatase, encoded by the cyp19 gene, converts androgens into estrogens. Modulation of aromatase CYP19 expression and function can cause alteration in the rate of estrogen production, disturbing the local and systemic levels of estrogens (Nagahama, 1994; Nakamura *et al.*, 1998; Strüssmann and Nakamura, 2002).

There are some reports on the effect of phytochemical extracts such as quercetin, genistein and diadzein (Dabrowski *et al.,* 2004; 2005; de Oca, 2005), which did not significantly affect the sex ratio of Nile tilapia. El-Sayed *et al.* (2012) evaluated the action of daidzein and genistein in soyabean meal used as protein source on 17α–methyltestosterone sex reversal treatment of Nile tilapia larvae. After 28-day trial these authors reported that the fish fed high inclusion levels of diadzein and genistein (10 to 30mg/kg) had an increased percentage of female with increasing elevated level of the phytoestrogen from 24.5 to 47.8% and from 17.5 to 42.5% respectively. They concluded that diadzein and genistein have a significant estrogenic effect on sexual differentiation of Nile tilapia larvae, and thus weakened methyltestesterone masculinization activity.

Ruksana *et al.* (2010) tested the efficacy of an aluminium laden compound, exemestane (EM), which is considered to be an aromatase inhibitor, on sex differentiation in genetically female Nile tilapia. These authors concluded that all females of the Nile tilapia treated with high levels of EM (1000 and 2000 µg/g of feed) during the critical developmental period for sex differentiation, developed testes. In addition they reported that the sex reversed males appeared to be sexually functional, because they had testes with efferent ducts and with all the stages of spermatogenesis (i.e. from primary spermatogonia to spermatozoa).

This study therefore, hypothesized that a diet containing natural estrogenic / androgenic compounds will affect the sexual differentiation and gonadal activity of sexually undifferentiated tilapia fry and disturb the sex ratio in favour of a particular sex (i.e. male or female).

6.2 Materials and Methods

The trial was conducted in a recirculating aquaria system (RAS) at the Welgevallen Experiment Farm, Stellenbosch University, South Africa (see Chapter 3, for more detail on the facilities and management). The Geographical Position System (GPS) coordinates are 33°56' 33.95" S and 18°51'56.15". The facility consist of 72 glass tanks, each tank with a dimension of 58 x 57 x 40 cm with water holding capacity of 94 litres.

The study was conducted between June and October 2011 consisting of two trials, the first for which Pawpaw Seed powder (P) was included in a commercial tilapia starter feed (Basal Diet, BD), and fed to first exogenous feeding Mozambique tilapia (*Oreochromis mossambicus*) fry, after the absorption of yolk sac.

During the second trial Moringa Seed powder (M) was included in the Basal Diet, and fed to first exogenous feeding *O. mossambicus* fry. The experimental design for trial 1 and 2 is presented in Tables 6.1 and 6.2 respectively. A negative control (NC) treatment comprising of the Basal Diet, and Positive Control containing 17α-Methyltestosterone (MT), were included in both parts of the study. The details of the preparation of the Pawpaw seed powder and Moringa seed powder and the experimental diets are presented in Chapter 3.

Table 6.1 The experimental design (3 x 4 = 12 factorial) for the assessment of the effect of 17α-Methyltestosterone and Pawpaw Seed meal on sexual differentiation in sexually undifferentiated post hatch tilapia fry, *Oreochromis mossambicus*.

Treatments	Designation	Number of fish	Dosage (g/kg of Basal Diet[1])
Negative Control	$NC_{(I-IV)}$	4x200	No dosage
Positive Control	$MT_{(I-IV)}$	4x200	60mg 17α-Methyltestosterone
Pawpaw Seed	$P_{(I-IV)}$	4x200	15g Pawpaw Seed powder

[1] Basal Diet: AQUANUTRO Tilapia starter diet (Nutroscience (Pty) Ltd, SA). Replicates per treatment = 4

Table 6.2 The experimental design (3 x4 = 12 factorial) for the assessment of the effect 17α-Methyltestosterone and Moringa Seed meal on sexual differentiation in sexually undifferentiated fry (9-12 day post hatch) of the tilapia, *Oreochromis mossambicus*.

Treatments	Designation	Number of fish	Dosage (g/kg of Basal Diet[1])
Negative Control	$NC_{(I-IV)}$	4x200	No dosage
Positive Control	$MT_{(I-IV)}$	4x200	60mg 17α-Methyltestosterone
Moringa Seed	$M_{(I-IV)}$	4x200	15g Moringa Seed powder

[1] Basal Diet: AQUANUTRO Tilapia starter diet (Nutroscience (Pty) Ltd, SA). Replicates per treatment = 4

A detail description of the experimental procedures and data recording has been provided in Chapter 3, including selection of breeders and fry production, stocking of fry (sexually undifferentiated fish 9 – 12 day-post-hatch), feeding, water quality and maintenance and data collection (i.e. survivorship and sex ratio).

In this experiment III, after the 30 trial, fish were fed on basal diet for another 60 days. At the end of the 90 day experimental period (i.e. 30 days treatment and 60 days control diet) gender could be comfortably determined in the fish; so), 50 fish were randomly sampled from each of the 4, replicates equal to 200 fish per treatment and sex determined.

Gender was determined by the presence/absence of specific external morphological characteristics, i.e. genital papillae, and the length and shape of the dorsal and anal fins. Gender was confirmed by gonadal examination, through dissection. The total number of males and females identified after dissection were recorded. Also, 4 fish (2males and 2 females) were randomly taken from each group of 50 fish, and the total length, body depth and body weight measured, and their gonads fixed in Bouin's solution.

Mortality was monitored and recorded for all the experiments and the data was used to calculate survival rates. Dead fish were removed from the tank on daily basis as a standard management procedure

Data recorded during the study were captured and analysed in Microsoft Excel 2010. A Chi-square analysis was performed to test for the significance of deviation from the expected 1:1, male: female sex ratio (Bhujel, 2008) at a 5% probability level for each replicate group as well as for the pooled data for each treatment. Using the statistical programme, SAS Version 9.1, a one-way analysis (ANOVA) was performed to test for the significance of variance in the deviation from the 1:1 ratio, at $P<0.05$.

Survival rate was determined for each treatment group, as well as the pooled data for each treatment, expressed as the mean ± sd according to the equation below:

$$\text{Survival Rate (\%)} = \frac{\text{Final Number of Fish}}{\text{Initial Number of Fish}} \times 100$$

(Source: Huang and Chin, 1997, modified)

6.3 Results

6.3.1 Effect of treatment on sex ratio

The observed sex ratio of the *O. mossambicus* fingerlings fed Pawpaw Seed meal at a concentration of 15g/kg Basal Diet over 90-days is presented in Table 6.3 and Figure 6.1. Out of 200 fish examined per replicate fed the Pawpaw Seed treatment a total of 65.0 ± 4.58 were classified as males on the basis of gonadal assessment (Figure 6.1). Pawpaw Seed (P) had a significant effect on the sexual differentiation of *O mossambicus* fry with the chi-square value against the expected 1:1 sex ratio of 18.0 ($P_{(<0.05)} = 3.841$).. Neither the Positive Control (MT) nor the Negative Control (NC) induced any significant deviation, as confirmed by the respective Chi-square values. In the Positive Control group a total of 10 fish from the 200 sampled were found to possess both testicular and ovarian tissues, whilst two such cases were found amongst 200 fish sampled in the Negative Control group, and none from the Pawpaw Seed treatment group.

Table 6.3 The male:female sex ratio in a mixed sex population of tilapia (*Oreochromis mossambicus*), after a 90 day treatment as Negative Control (CT), Positive Control (MT) and Pawpaw Seed (P).

Treatment	Male	Female	Intersex	Total Fish Examined	Sex Ratio	Calculated χ^2	$P_{(0.05;\,1)}$ $\chi^2=3.841$
NC	107	91	2	200	1.18:1	1.30	NS
MT	104	86	10	200	1.08:1	0.32	NS
P	130	70	0	200	1.88:1	18.00	S

The observed sex ratio of the *O. mossambicus* fingerlings fed Moringa Seed meal at a concentration of 15g/kg Basal Diet, over a 90-day trial period, is presented in Table 6.4 and Figure 6.2. In this trial both the Moringa Seed (M) and the Positive Control (MT) treatments showed a significant effect on the sex ratio in developing tilapia *O mossambicus*. From the 200 fish examined from fish fed M treatment group an average

of 65.50 ± 4.12 were classified as males (Figure 6.2), confirming a significant deviation from the expected 1:1 sex ratio against the Chi-square value of $P_{(<0.05;\ 1)}$ =3.841, and p-value < 0.001.

Table 6.4 The male:female sex ratio in a mixed sex population of tilapia (*Oreochromis mossambicus*), after a 90 day treatment as Negative Control (NC), Positive Control (MT) and Moringa Seed (M).

Treatment	Male	Female	Intersex	Total Fish Examined	Sex Ratio	Calculated χ^2	$P_{(0.05;\ 1)}$ χ^2=3.841
NC	90	110	0	200	0.82:1	2.00	NS
MT	143	57	0	200	2.51:1	36.98	S
M	131	69	0	200	1.90:1	19.22	S

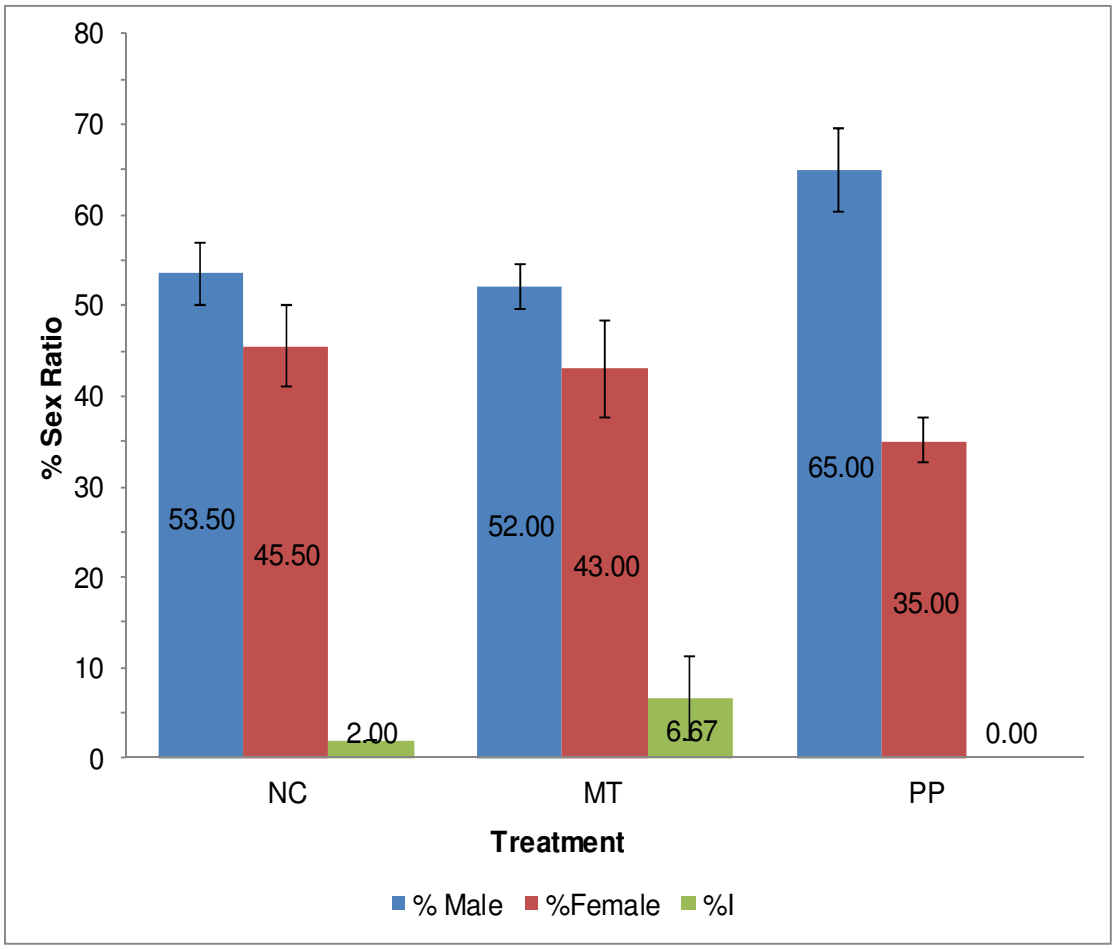

Figure 6.1 The Sex ratio (percentage) of Tilapia *O.mossabicus* at age 90 days over 4 replicates and 3 treatments Negative Control (NC), Positive Control (MT) and Pawpaw Seed (PP)

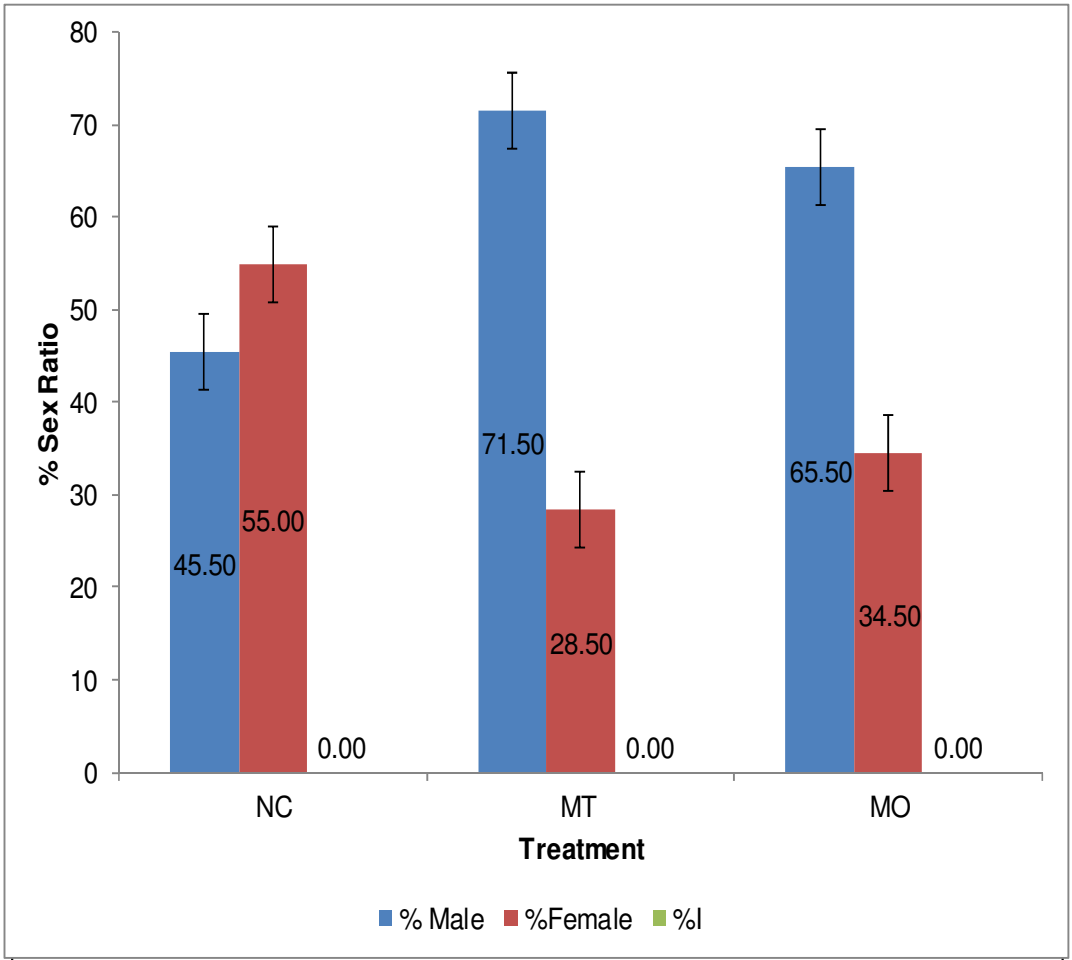

Figure 6.2 The sex ratio (percentage) of Tilapia (O. *mossambicus*) at age 90 days over 4 replicates and 3 treatments: Negative Control (NC), Positive Control (MT) and Moringa Seed (MO).

None of the gonads from sampled fish amongst any of the treatments displayed both testicular and ovarian tissue as in the case of the first trial.

6.3.3 Effect of treatment on survival rate

The effect of the treatments on survival during the first trial is shown in Table 6.5. The highest percentage survival was found in the Negative Control group at 97.88 ± 2.29, although not significantly different from any of the other treatments at $P<0.05$.

Table 6.5 Survival rates of Tilapia (*O. mossambicus*) over 90-day treatment as Negative Control (NC), Positive Control, (MT) and Papaw Seed (P), with 4 replicates per treatment.

Treatment (Diet)	Initial Number	Final Number	Survival (%± sd)
Negative Control (NC)	4x200	783	97.88 ± 2.29[a]
Positive Control (PC)	4x200	774	95.75± 3.28[a]
Pawpaw Seed (P)	4x200	772	96.50 ± 2.12[a]

[a] Values with similar superscript are not significant, Chi-square p-value, at P<0.05, 1df

Significant differences in survival rate were recorded during the 2nd trial in relation to the M treatment groups with an average of 91.00 ± 6.70, compared to that of the Negative and Positive Control groups, as reported in Table 6.6.

Table 6.6 Survival rates of Tilapia (*O. mossambicus*) over 90-day trial treatment as Negative Control (NC), Positive Control, (MT) and Moringa Seed (M), with 4 replicates per treatment

Treatment (Diet)	Initial Number	Final Number	Survival (%± s.d)
Negative Control (NC)	4x200	698	87.25 ± 5.32[a]
Positive Control (PC)	4x200	697	87.13± 5.51[a]
Moringa Seed (M)	4x200	728	91.00 ±6.70[b]

[a ,b] Values with different superscript are significant and same not significant, Chi-square p-value, at P<0.05, 1df

6.4 Discussion

Pawpaw and Moringa seed contain a broad spectrum of phytochemicals including polysaccharides, vitamins, minerals, enzymes, proteins, alkaloids, glycosides, fats and oils, lectins, saponins, flavonoids, sterols and several phenolics (Krishna *et al.*, 2008, Kumar *et al.*, 2010). Both plant species are highly valued food crops in SSA, with additional application in several traditional medicines (Raji *et al.*, 2005; Anwar *et al.,* 2007; Bose, 2007; Krishna *et al.*, 2008). The choice of Pawpaw and Moringa seeds for experimental treatments of tilapia stem from their known effect as a natural reproduction inhibitor, and it is readily available year round in Sub-Saharan Africa (SSA). Pawpaw and Moringa have been tested on animal models, such as mice and rats, showing antifertility properties (Krishna *et al.,* 2008; Kumar *et al.,* 2012). Some attempts have been made to use crude Pawpaw Seed as antifertility agent on sexually matured Nile tilapia to control its reproduction, with results which indicate that Pawpaw can interfere with breeding in tilapia (Ekanem and Okoronkwo, 2003; Jegede and Fagbenro, 2008; Abbas and Abbas, 2011).

Exogenous first feeding of tilapias starts at 9-12 days post hatching following depletion of the yolk (Rana, 1988) with no differentiation of the gonads. Sexual differentiation is known to occur in the genus *Oreochromis* fry at or around 17-19 days after hatching (Yamazaki, 1983; Mair and Little, 1991; El-Sayed, 2006). It is therefore, possible to intervene at this early stage of development in the fish to direct gonadal development to produce monosex populations. Pandian and Varadaraj (1987) reported that in the Mozambique tilapia, *O. mossambicus* the period where the fry will be most sensitive for such manipulation is in the period 10 to 20 days (i.e. at about 15 - 30mm length) of the post hatching period. The morphological parameters (i.e. length and weight) and age used in this study conform to the assertion that a successful sex change or reversal requires newly hatched fry of length with a range 9-12mm, presumed to have sexually undifferentiated gonads (Green and Teichert-Coddington,1993), and a body mass in the range of 10mg to 30mg (i.e. 0.0010g -0.0030) (Phelps, 2006). According to Nakamura *et al.* (1998) gonadal sex differentiation in teleost fish is affected by several exogenous factors such as sex hormones, temperature, and pH. Therefore, applying any of these parameters mentioned above during the critical period of sexual differentiation will affect the direction in which the phenotypic sex of the fish could be expressed.

The use of phytochemicals as a possible alternative to 17MT has received much attention since the estrogenic activity of flavonoids in fish diet was reported by Pelissero *et al.* (1991). Notable attempts on the use of phytochemicals to induce sex change of tilapias have been reported by Dabrowski *et al.* (2004; 2005) and; de Oca (2005). These authors used the refined phytochemicals that included quercetin, genistein and diadzein (Pelissero *et al.*, 1996), and reported that the phytochemicals did not significantly affect the masculinization of tilapia. De Oca (2005) indicated that pure genistein and quercertin did not affect the sex ratio of genetically all-female Nile tilapia, by obtaining equal female to male ratio compared to control group of 97% female. Green and Kelly (2009) investigated the potential of genistein to alter phynotypic sex during sexual differentiation in channel catfish and reported an increase in the proportion of phenotypical male after long exposure to genistein. These studies provided the preliminary insight on the *in vivo* response to pure phytochemicals when dietary administration was used in sexually undifferentiated fish.

This study used crude Pawpaw and Moringa Seed powder to investigate the potential of these biochemical plant components, acting as endocrine disrupters, to influence the direction gonadal sex differentiation in Mozambique tilapia (*Oreochromis mossambicus*). For each of the treatments, a significant increase was obtained in the percentage males of 65.0 ± 4.58 for Pawpaw and 65.50 ± 4.12 for Moringa.

Shelton (2006) intimated that the extensive use and success of sex reversal is largely based on the following assumptions (a) treatment must proceed during a critical period of gonadal differentiation, (b) steroids (androgens/estrogens) mimic natural induction by genetic sex-determining factors so as to alter development of the phenotypic or gonadal sex, (c) the exogenous steroid must be efficacious, adequately concentrated, and efficiently delivered so as to provide the physiological or pharmacological effect; (d) steroid-induced development of gonadal sex does not spontaneously revert, and, (e) genotypic sex is not affected by the phenotypic alteration treatment. The reliability of any chemical to induce effect on fish gonad will depend largely on the amount available for the fish to ingest and present at the site of estrogenic activity (Phelps,

2006; Shelton, 2006). The significant increase in the observed percentage of males is an indication of the presence of phytoestrogens in Pawpaw and Moringa seed and its effect on sexual differentiation in relation to the postulations of Shelton (2006).

The reported phytoestrogens identified in Pawpaw plant parts include β-sitosterol, saponins, and flavonoids (Krishna *et al*., 2008) and that of Moringa plant parts include saponins, oleanolic acid-3-glucoside and the flavonoids, quercetin and kaempferol (Anwar *et al*., 2007; Kumar *et al.,* 2010). The interaction of these phytoestrogens with the endogenous hormones of the fish could be seen as the cause of change in sex ratio and indicates that the dietary administration of Pawpaw or Moringa in the crude form could affect gonadal sex differentiation in juvenile tilapia.

The exact mechanism through which Pawpaw and Moringa seed could act at the cellular or molecular level have not been established, however two possible scenarios have been adduced for expression of phytoestrogens in endocrine modulation. Phytoestrogens that show close structural similarity to androgens and estrogens, have been shown under experimental conditions in *in vitro* studies to block the biosynthesis and action of estrogens by (1) inhibition of aromatase activity and other steroid metabolism related enzymes, or (2) by competition for the estrogenic nuclear receptors (α and β ER), that could possibly mimic the sex-reversal effects of androgen treatments in fish (Dabrowski *et al*., 2005; de Oca, 2005; Moutsatsou, 2007).

In the teleost fish ovarian differentiation commences at an earlier stage than as in testicular tissue. Sexual differentiation into ovarian cells occurs with biosynthesis of estradiol -17β from cholesterol, through enzymatic activity of aromatase (Nagahama, 1994; Nakamura *et al.,* 1998). Thus estrogenenic hormones are a product of aromatase, and thus any chemical compounds, such as the phytoestrogens (β-sitosterol, saponins, oleanolic acid-3-glucoside, genistein, quercetin and kaempferol) that can block the activity of aromatase, suppressing estrogen biosynthesis in cells (Pelissero *et al*., 1996; Dabrowski *et al*., 2004), which could switch ovarian development to testicular development. The studies of Dabrowski *et al.* (2005), de Oca (2005) and El-Sayed *et al.* (2012) give an indication that phytoestrogens could exert influences on gonadal differentiation of fish. It has not been established that, Pawpaw and Moringa are aromatase inhibitors, however, considering the fact that both plants contain the above known phytoestrogens with known aromatization activity, it can be postulated that both Pawpaw and Moringa are potential aromatizing agents.

In relation to the second scenario, *in vitro* studies have indicated that some phytoestrogens have structures similar to that of natural steroid hormones, thereby it can mimic, compete and displace endogenous estrogens from binding sites on estrogen receptors (Moutsautsou, 2007), consequently, they can act as antiestrogens or weak estrogens thereby eliciting their biological activity. According to Matthews *et al.* (2000) activity of estrogen in target cells manifest itself through binding to estrogen receptors. Based on the chemical structure phytoestrogens such as quercetin, genistein and diadzein, have a capacity to compete for binding sites and displace estradiol at the estrogen receptors (ERs) binding sites, thereby modulating the genetic expression of sexual orientation (Miksick, 1995; Morito *et al*., 2001; de Oca, 2005; Moutsautsou,

2007). Therefore phytoestrogens present in Pawpaw and Moringa, can elicit the observed effects in fish gonads.

The manifestation of phytoestrogen effect depends on factors which include introduction of the chemical to the fry that allow for interaction to occur during the critical period of gonadal differentiation; the capacity of phytoestrogens to mimic natural estrogen that can induce the genetic sex-determining factors to alter development of the phenotypic or gonadal sex and more importantly it must be efficacious, adequately concentrated, and efficiently delivered so as to provide the physiological or pharmacological effect (Shelton, 2006). The most important criteria to determine whether a natural or synthetic compound can successfully induce sex reversal, are a) the time of onset of treatment, b) the duration of treatment, c) the dose and type of hormones used, and d) the quantity of the hormone, or bioactive chemicals (Yamazaki, 1983; Nagahama, 1994; Nakamura *et al.*, 1998).

The success of sex-reversal techniques in tilapias to produce all-male populations has been found to depend on the intake of feed containing hormones which is in turn influenced by many factors (Mair and Little, 1991; El-Sayed, 2006). tTese including a) the quality of treated feed, composition of the raw ingredients, method of preparation and storage; b) palatability and particle size (both affect actual intake of first-feeding fry); c) feeding frequency and mode (e.g. ad libitum or, percentage of body weight and number of times feed is offered) and availability of treated food to all fry.

The two most recent studies involving the use of refined phytoestrogens- genistein and diadzein to induce sex reversal presented conflicting results. De Oca (2005) reported that genistein and diadzein could not masculinize genetically all-female Nile tilapia fish, whilst El-Sayed *et al.* (2012), indicates that genistein and diadzein decreased the masculinization effect of 17α-methyltesterone in genetically all-female Nile tilapia fish.

The results from both experiments indicated that the inclusion of Pawpaw and Moringa Seed had no negative impact on the survival rate of tilapia fry compared to Positive and Negative Control diets ($P<0.05$, Tables 6.1and 6.2). In fact the Moringa seed treatment yielded a significantly higher survival rate compared to the Positive and Negative Control diets ($P<0.05$).

El-Sayed *et al.* (2012) pointed to the importance of maintaining a good rate of survival during the production and supply of tilapia fry and fingerlings. The critical period of sexual differentiation in *Oreochromis* fry at 17-19 days after hatching (Yamazaki, 1983; Mair and Little, 1991; El-Sayed, 2006), is also the period where the fish is very fragile and susceptible to environmental factors, stress and disease.

6.5 Conclusions

The inclusion of the crude form of Pawpaw and Moringa seed, containing phytoestrogens such as genistein, quercetin, kaempferol, diadzein and, β-sisterol, resulted in up to 65% masculinization in a mixed sex

population of Mozambique tilapia fry. From these results it can be concluded that phytoestrogens in Pawpaw and Moringa seed affected sexual differentiation in first feeding fry. Further studies are, however, required to understand the exact mechanism through which the Pawpaw and Moringa seed exert their effect. It could be postulated, that the results are a manifestation of multiple actions of the various phytoestrogen compounds present in Pawpaw and Moringa seed, and not based on the single activity of one particular phytochemical. The biochemical composition of Pawpaw and Moringa Seed should be investigated further to establish the relative quantities and range of the phytoestrogens.

The results obtained provide an indication of the use of Pawpaw and Moringa as a possible alternative to induce sex reversal in mixed sex tilapia populations. This could be of particular importance to aquaculture development in rural areas of Sub-Saharan African countries, given the abundant year round availability of these compounds. Further studies are required to optimize dosage levels for Pawpaw and Moringa Seed, in terms of concentration, duration and methods of application, as well as the interaction of the treatments with key environmental factors such as water temperature.

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Chapter 7

Determination of the Triterpene Acids (Oleanolic Acid and Ursolic Acid) in Pawpaw Seed Meal, Moringa Seed Meal and Fish Tissues

Abstract

The study attempted to quantitify the levels of triterpene acids, oleanolic acid (OA) and ursolic acid (UA), in Pawpaw and Moringa seed powder through the use of ultra-high performance liquid chromatography with electrospray ionization mass spectrometer detection (UHPLC-ESI-MS/MS). The oleanolic acid and ursolic acid are isomeric compounds that are difficult to separate. Studies have shown that both oleanolic acid and ursolic acid possess antifertility property. Only OA was found in Moringa seed. None of the triterpene acid was found in the Pawpaw seed. The precence of oleanolic acid in the Moringa seed powder was confirmed at a concentration level of 0.508µg/g ± 0.032. Analysis need to be extended to include Moringa seed from various parts of Sub-Saharan Africa to establish the range of oleanolic acid and ursolic acid composition. The presence of oleanolic acid explains the observed effect of Moringa Seed powder on sexual development and differentiation in tilapia with the potential for application in controlling the problem of precocious breeding in tilapia culture in small scale farming systems in Sub-Saharan Africa.

7.1 Introductions

7.1.1 Rationale for Use of Phytochemicals

The early maturation and habitual spawning associated with mixed tilapia culture remains a challenge to the expansion of tilapia production in Sub-Saharan African (SSA) countries such as Ghana. For commercial and profitable culture to be fully embraced by resourced-poor rural farmers in SSA, the problem of prolific breeding and stunted growth associated with tilapia production has to be addressed.

The monsex culture i.e. maintaining only male tilapia in a culture system is a potential way to address the above mentioned problems, and is presently the preferred cultivation system on commercial farms (Popma, and Lovshin, 1995). The use of hormones to manipulate the sexual differentiation of tilapia to yield all male populations is the common technology that is used today, but the technology is not readily available to majority of SSA countries. Despite elimination of such hormones from fish on cessation of treatment to non-detectable levels after 90 days, and in fish harvested after 6 months (Green and Teichert-Coddington, 2000; Fitzsimmons, 2007), the use of such hormones is still perceived as a potential health risk by fish consumers in developed countries (Lückstädt *et al*., 2006). Considering the potential health and environmental risks associated with the use of hormones (Dabrowski *et al*., 2005; Lückstädt *et al.,* 2006), and the shortcomings of existing methods and techniques discussed by Mair and Little (1991), alternative methods to control

undesirable tilapia recruitment in ponds needs to be investigated, such as the use of natural reproduction inhibitors found in plants .

Medicinal plants have the potential to be used for the manipulation of gender differentiation in tilapia, due to the fact that they contain bioactive components that have an antifertility action in animals. Some of these plants have been used successfully to induce sterility in laboratory animals (Bodharkar *et al.*, 1974; Das, 1980), and also as human antifertility agents in traditional medicine treatments in various countries (Kumar *et al.*, 2012). Pawpaw (*Carica papaya*) seed, administered orally to male albino rats, effectively controlled their reproduction (Udoh and Kehinde 1999) and reduced sperm motility (Pathak *et al.*, 2005). Aqueous extracts of the root and bark of *Moringa oleifera* are effective in preventing implantation in rats (Shukla *et al.*, 1989) with the ethanolic extract of *M. oleifera* leaves indicating 100% abortifacient activity in female albino rats (Charles Foster strain) (Nath *et al.*, 1992). Anwar *et al.* (2007) in a review reported that the root of *M. oleifera* has antifertility properties, with the root and flowers containing the abortifacient compounds.

There is therefore some merit in the assessment of similar effects on reproduction of tilapia. These studies suggest that a contraceptive efficacy of dietary plant nutrients with antifertility or abortifacient activity could be used by resourced-poor producers to combat problems of tilapia precocious breeding in production ponds.

7.1.2 Bioactive compounds with abortifacient / antifertIlity properties

Kitts (1994) defined bioactive compounds as “extranutritional constituents that typically are naturally occurring in small quantities in plant products and lipid rich foods”. However, according to Bernhoft (2010), the classical definition of bioactive compound is that “it is secondary plant metabolites that have pharmacological or toxicological effects in man and animals”. The author further states that bioactive compounds can be either beneficial or detrimental to animals depending on the quantity eaten and the content of intake. The secondary metabolites that are produced after ingestion of plants that contain antifertility compounds, have been found to have important functions in the living plants which produce them. For example, flavonoids can protect the original plant against free radicals generated during photosynthesis. Terpenoids may attract pollinators or seed dispersers, or inhibit competing plants. Alkaloids usually ward off herbivorous animals or insect attacks (phytoalexins). Plants produce astounding varieties of these secondary plant metabolites, which are now being exploited for their potential inclusion as feed additives in livestock and aquaculture feeds (Francis *et al.*, 2002; Makkar *et al.*, 2007).

Phytogenic feed additives are plant-derived products or compounds incorporated into animal feeds to improve productivity of livestock, swine and poultry through amelioration of feed properties and food quality, and the optimization of overall production performance. Such additives are claimed to exert antioxidative, antimicrobial, and growth-promoting effects in livestock, which can potentially be associated with improved feed consumption, supposedly because of improved palatability of the diet (Windisch *et al.*, 2007; Scheurmann *et al.*, 2009). Steiner (2009) summed “phytogenics as a term that refers to group of natural

growth promoters (NGPs) or non-antibiotic growth promoters (NAGPs) derived from herbs, spices and other plants". For instance, saponins, are considered as a special class of phytogenic substances, because they are able to reduce intestinal ammonia (NH_3) and hence alleviate an important stress factor to animal health (Francis *et al*., 2002a; 2002b). Phytogenics are also being tried as a replacement for fish meal in aquaculture feeds (Francis *et al*., 2002b; Makkar *et al*., 2007).

Phytochemicals known as phytoestrogens (such as isoflavonoids, flavonoids and saponins) are natural estrogenic or estrogenic compounds found in plants such as soy, tea, fruits and vegetables that present an anti-estrogenic or estrogenic activity (Pelissori *et. al*, 1991a; 1991b; Pelissori and Sumpter, 1992; Dabrowski *et al.,* 2004). Phytoestrogens are structurally or functionally similar to or mimic mammalian estrogens and can thus have an effect on the sexual differentiation of fish.

Studies have identified some phytochemicals such as the triterpene acids- oleanolic acid (OA), ursolic acid (UA) (Rajasekaran *et.al*, 1988), and their derivatives e.g. glycoside (Das, 1980), oleanolic acid-3 β-glucoside and oleanolic acid 3-β-D-glucuronide (Das *et al*., 2011), saponin (steroidal or triterpenoid glycoside) (Makker *et. al*., 2007; Souad *et al*., 2007) isolated from plants as possessing abortifacient properties and thus have the potential to be used as an antifertility treatment. Oleanolic acid and ursolic acid are hydroxyl pentacyclic triterpenoic acids (HPTAs), considered as ubiquitous triterpenoids in plant kingdom (Liu, 2005). Oleanolic acid and UA are isomers with similar chemical structures and often exist simultaneously in the same plant as aglycones of saponin and as free acids (Janicsák *et al*., 2006). These isomeric triterpenic acids occur mostly in medicinal herbs and plants and naturally in a large variety of vegetarian foods forming an integral part of the human diet (Liu, 2005; Furtado *et al*., 2008). According to Zhou *et al.* (2011) the distribution of oleanolic acid and ursolic acid in plants vary greatly between different families, genera and species.

Rajasekaran *et al.* (1988) tested antifertility activity of oleanolic acid isolated from the flowers of *Eugenia jambolana* in male albino rats. They reported that oleanolic acid arrested spermatogenesis but did not cause any abnormality to spermatogenic cells, Leydig cells and Sertoli cells in the rats. In a review of oleanolic acid, Liu (2005) reported an antifertility effect in rats with oleanolic acid also inhibiting testosterone 5α-reductase activity. In a study by Mdhuli and Van der Horst (2002), oleanolic acid was administered orally to a male Wister rat, for 30-days followed by a 14 day withdrawal period. Their study indicated that oleanolic acid induced reversible sterility in the male Wistar rats, without any adverse effects on *libido*.

Ursolic acid has been found to be the most abundant triterpenic acid in the leaves of the plant *Ocimum sanctum* (Rajasekaran *et al*., 1980; Raja, 2012). Ursolic acid has been shown to have the potential of inhibiting sperm motility (Chattopadhyay *et al*., 2005), and also possess antifertility activity in rats and mice (Raja, 2012).

Das *et al.* (2011) evaluated the spermicidal activity of oleanolic acid 3-β-D-glucuronide (OAG), an active principle isolated from root extracts of *Sesbania sesban*, which showed some significant spermicidal activity.

The antifertility effect of these triterpene acids is attributed to their antiestrogenic effect which may be responsible for arresting spermatogenesis (Das *et al*., 2011; Raja 2012) in the tested animal models.

7.1.3 Methods for determination of triterpene acids

Bioactive compounds such as oleanolic acid and ursolic acid are usually present as a minor component of a plant extract. Because oleanolic acid and ursolic acid are isomers with similar chemical structures (see Chapter 2, section 2.6.3.7, Figure 2.12), they are very difficult to separate. According to Liang *et al.* (2009) a number of techniques and methods have attempted to quantitatively separate the tritepene acids in different plant materials over the past decades. Some of the methods include gas chromatography (GC), high-performance liquid chromatography (HPLC), cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC), micellar electrokinetic capillary chromatography (MECC) and nonaqueous capillary electrophoresis (NACE). These authors emphasised that each of the method have bottlenecks, for insntance in GC, sample preparation is time consuming and difficult, and a need for pre-column treatment. In the CD-MEKC, MECC and NACE methods, there is a complicated buffer system with low reproducibility. They concluded that HPLC, using a mobile phase consisting of an aqueous acid buffer is preferably the method used to determine oleanolic and ursolic acid.

7.1.4 High Performance Liquid Chromatography (HPLC)

According to Kellner *et al.* (2004) the high performance liquid chromatography (HPLC) is the method used to separate unipolar and isomeric compound. The HPLC technique is a unique, which is highly adptable in its usage, and now used in isolation of natural products. Current, it is the method of choice in the study of quality of herbal plant products (Fan *et al.,* 2006; Sasidharan *et al*., 2011). The HPLC method allows for expeditious separation of multicomponent samples on both preparartuve and analytica scles.

The basic bench top HPLC instrument design consist of a solvent delivery pump, a sample introduction device which could be an auto-sampler or manual injection valve, an analytical column, a guard column, detector and a recorder or a printer (Kellner *et al*., 2004; Swartz, 2005; Sasidharan *et al*., 2011). Separation of chemicals by means of HPLC is based on the principle that in a particular column and mobile phase different compounds have different migration rates. Kellner *et al.* (2004) emphasised that the magnitude of separation depend mostly on the choice buffer solutions used for the stationary phase and mobile phase. In general, the mobile phase system in HPLC use a single unchanged solution (i.e. isocratic system), for identification and separation of phytochemicals (Kellner *et al*., 2004; Swartz, 2005; Sasidharan *et al*., 2011).

Identification of compounds using HPLC involve test running a HPLC assay. A proper HPLC that can be used to separate closely related samples must have the right mobile phase system, constant flow rate, suitable detectors and appropriate column. Each compound should have a characteristic peak under certain chromatographic conditions. Developing a separation assay involves selecting a detector and setting it at optimal detection levels. In separation assay, a recognised peak of the known sample is observed on the

chromatograph, with its own retention time and separated from other peaks at the detection levels on which the assay is being performed (Kellner *et al.*, 2004; Swartz, 2005).

One of the established rapid, convenient and reliable method to separate and quantify oleanolic acid and ursolic acid is reversed-phase high performance liquid chromatographic (RP-HPLC). Reverse-phase has been used to separate and quantify oleanolic acid and ursolic acid in the following plants which include: *Macrocarpium officinalis* (Wang *et al.*, 2008); *Oldenlandia diffusa* (Liang *et al.*, 2009); leaves of *Olea europaea*, *Salvia officinalis*, *Thymus vulgaris, Origanum majorana* (Kontogianni *et al.*, 2009) and *Eriobotrya japonica* Lindl (Xu *et al.*, 2012).

Some of the proven detectors which have been used in phytochemical analysis include ultra-violet (UV), diode array detector (DAD) coupled with mass spectrometer (MS) (Tsao and Deng, 2004; Kellner *et al.*, 2004). The suitability of a UV detector is determined by the fact that the majority of naturally occurring compounds have some UV absorbance at low wavelengths (190-210 nm) and UV has a high sensitivity capable of detecting minute amounts of bioactive chemicals within a sample (Lia *et al.*, 2004).

7.1.5 Current Forms of High Performance Liquid Chromatography

The high performance liquid chromatography has proven to be a useful technique in analytical science. According to He (2000), liquid chromatography coupled with mass spectrometry (LC/MS) is one of the powerful techniques for the analysis of complex botanical extracts. It is able to provide relevant information making it possible to identify structurally related compounds. Therefore, a combined HPLC and MS technique ensure rapid assessment and a reliable identification of bioactive compunds in medicinal herbs, particularly, where there is no pure standard (Cai *et al.*, 2002; Ye *et al.*, 2007).

A new category of HPLCs are the high performance liquid chromatography-electrospray ionization-mass spectrometer detection (HPLC–ESI-MS) and ultra-high performance liquid chromatography-electrospray ionization- tandem mass spectrometry (UHPLC-ESI-MS/MS). According to Swartz (2005) UPLC-ESI-MS/MS retains the basic principles and the practicality of HPLC, whilst increasing the overall interlaced attributes of speed, sensitivity and resolution. The latter method is now considered to be the most powerful method for the separation, quantification and structural determination of position isomers with very similar structures, such as, oleanolic acid and ursolic acid in a variety of botanical raw materials (Makarov *et al.*, 2009). According to Voutquenne *et al.* (2003) oleanolic acid gives a quasi -molecular ion at *m/z* 455, [M-H]- in ESI negative mode. The UPLC-ESI-MS/MS technique has been utilised by several investigators such as Wang, *et al.* (2008), Liang *et al.* (2009), Scheidner *et al.* (2009), Csuk and Siewert (2011), Pollier and Goossens (2012), and Zhang *et al.*, (2012) for separation and quantification of oleanolic acid and ursolic acid.

Owing to the difficulty associated with the separation of the steromeric isomers, the study used UPLC-ESI-MS/MS to rapidly and accurately determine the levels of oleanolic acid and ursolic acid in Pawpaw and

Moringa seed powder, which has been used to separate dyes (Li *et al.*, 2010), and also in pharmacokinetic studies (Han *et al.*, 2010; Liu *et al.*, 2012).

7.2 Material and Methods

7.2.1 Methodology and Location

Pawpaw and Moringa seed powder samples were analysed at the Central Analytical Facilities, Mass Spectrometry Unit (CAF-MSU), in Stellenbosch University. In addition, fish meat tissue samples were also analysed to determine whether and how much of the active compounds were incorporated in the carcasses of the fish fed diets containing the Pawpaw and Moringa seed powder.

7.2.2. Pawpaw and Moringa Seed Powder

The processing and preparation of the respective samples (i.e. Pawpaw and Moringa seed powder) analysed in this part of the study is discussed in detail in Chapter 3, section 3.3.1.1 and 3.3.1.2.

7.2.3 Fish Tissues Samples

Two fish were randomly sampled from the treatment groups that were fed 5.0, 10.0 and 15.0 g/kg base diet (BD) of Pawpaw seed powder and Moringa seed powder. Each fish sampled was placed in sealed plastic bag, labelled and stored in a -20^{o}C fridge. Problems were experienced with the cold storage of the samples beyond project control, in that the freezing unit defrosted causing the unrecorded thawing of the samples. Only the head region and the fillets components could therefore, be used for the analysis for the presence of the triterpene acids. Prior to being analysed, each fish was descaled, and the carcass separated into the head and the rest of the body. The skin and internal organs were removed, where after the head and fillet components were ground using a blender (RUSSELL HOBBS, 1000W Satin Blender with 1.5 litre jug capacity). For ease of grinding, 100mL of distilled water was added gradually in the process. Wet samples were freeze dried at -80^{o}C, and stored at -20 ^{o}C until further analysis.

7.2.4 Reagents and Chemicals

The purified forms of oleanolic acid and ursolic acid were obtained from Sigma-Aldrich (Gmbh, D-91625 Schnelldorf, Germany). The other media required for analysis (ethanol, methanol, acetonitrile and chloroform) were also from (Sigma-Aldrich, Pty). Purified water which was filtered from Milipore water purification system was used throughout the experiment.

(a) Preparation of Standard Solutions

A stock solution of oleanolic acid and ursolic acid was prepared in 950 µL methanol and 50 µL dichloromethane for easy and complete dissolution, to obtain 150mg/L or ppm and stored at 4 ^{o}C. Between 6 and 10 sets of the stock solution were prepared beforehand, and used as required.

Calibration standards (0.0012, 0.0024, 0.012, 0.06, 0.12, 0.06 mg/L) were prepared from the stock solution by serial dilution of methanol (Wang *et al.*, 2008). A standard solution for recovery was prepared through methanol serial dilution with final concentration of 12.0mg/L. From this solution 50 µL with a final concentration of 0.15mg/L after a 10 times concentration of the sample was used to spike the extracting materials for the efficiency of extraction procedure.

(b) Selection of Solvents for Extraction

Factors determining analyte recovery include the affinity of the compound to be analysed for the solvent, the solvent to sample volume ratio, and the number of extraction steps. As this was a first attempt to determine oleanolic acid and ursolic acid in Pawpaw and Moringa seed, and fish tissues, a number of solvents were tested for effective extraction. More so, it is known that oleanolic acid and UA in nature coexist in micro quantities, yields are usually very low (Rajasekaran *et al.*, 1988), and show almost the same solubility in different solvents, depending on the accuracy of the measurements (Schneider *et al.*, 2009). Owing to their similar molecular structure differing only in the position of two methyl groups (see Chapter 2), their extraction and separation always pose a challenge (Cheng *et al.*, 2011).

The following extraction solvents were attempted which included:

a) Acetonitrile (modified from Sharma *et al.*, 1997)
b) Mixture made up of 50% Acetonitrile and 50% Chloroform (modified from Wang *et al.*, 2008)
c) Mixture of 95% Ethanol and 5% Pure water (modified from Xia *et al.*, 2012)
d) Mixture of 50% Acetonitrile, 45 % Methanol and 5% Chloroform (modified from Wang *et al.*, 2008 and Xia *et al.*, 2012)

Each solution was tested twice, for its efficacy as an extraction solvent following modified procedures as used in Sharma *et al.* (1997); Wang *et al.* (2008); and Liang *et al. (*2009). The extractants were analysed following standard procedures as described below, with the solvent consisting of 50% Acetonitrile, 45 % Methanol and 5% Chloroform giving the best recovery rate. This solvent was therefore used for all subsequent extractions.

(c) Selection of the Mobile Phase

To find the optimal elution conditions, two mobile phase systems composed of simple buffers were tested. The buffers were chosen on the basis of conformity and usage with respect to calibration and specifications of mobile phase in CAF-MSU UPLC-MS instrumentation. The first consisted of 0.1% formic acid-acetonitrile (25:75, v/v). This could not separate the standard oleanolic and ursolic acids. Another mobile phase made up of a buffer of 10mM ammonium acetate and 100% methanol at a ratio of 25:75, v/v was tested. The second buffer combination was able to give clear separation of the two triterpene acids (Figure 7.1). Thus the mobile phase used in this experiment for the LC-MS analysis consisted of 10mM ammonium acetate and 100% methanol, at a ratio of 25:75, v/v.

7.2.5 Extraction Procedure

The dried powder (1.0 g) of the respective specimen (Pawpaw or Moringa seed powder; fish tissues: head region and fillet) was accurately weighed using a RADWAG 2007 scale (Model WLC1/A1, RADWAG®). Each 1.0g of powdered sample was placed in a capped 50mL centrifuge plastic tube and 20mL of extraction solvent (i.e. 50% Acetonitrile, 45 % Methanol and 1% Chloroform, v/v/v) was added at a liquid to material ratio of 20:1 (Xia *et al*., 2012). The mixture was mixed thoroughly for 50-60 seconds by a Vortex mixer (VORTEX GENIE 2, Model G-560 E, Bohemia, NY, USA). The mixed samples were then transferred to 500mL flat-bottom flask (Erlenmeyer) and immersed in the water bath component of the sonication device (Sonication Bath, Lasec Lab & Scientific Equipment). Sonication was done for 30minutes whereafter the samples were centrifuged at 7500rpm, 4^{o}C for 20 minutes (Eppendorf Centrifuge 5430R, Eppendorf AG 22331, Hamburg, Germany). After extraction and centrifugation, the supernatant was aspirated. The extraction process was repeated to obtain a solution of 35-40 mL before being dried (Wang *et al*., 2008). For the drying out of the samples about 5-7 mL of the collected supernatant was repeatedly transferred into 14mL centrifuge plastic bottle and exposed to a stream of nitrogen (N_2) gas until completely dried. The dried sample was stored at 4^{o}C, until later analysis. The samples were resuspended in 4mL methanol before UPLC-MS analysis.

7.2.6 UPLC-ESI- MS/MS analysis

Oleanolic acid and ursolic acid were analysed using ultra performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS). The separation was carried out with a Waters ACQUITY Bridged Ethyl Hybrid, (BEH) C18 1.7µm, 2.1 x 100 mm column. OA and UA were separated using an isocratic elution mobile phase consisting of 10 mM ammonium acetate-methanol (25:75, v/v) at a flow rate of 0.38 mL/min and at a column temperature of 50 ^{o}C.

The Xevo TQ (Waters Corporation, Milford, MA, USA) was operated in ESI negative mode and the m/z 455 was monitored (the $[M–H]^-$ ion for both OA and UA). Although oleanolic acid and ursolic acid showed no significant collision-induced dissociation (CID), the MS was still operated in multiple reaction monitoring (MRM) mode, monitoring the transition from m/z 455 but to 455 to minimize non-specific peaks. The cone voltage and collision energy were x and y respectively.

The peak area was used to calculate the amount of oleanolic acid and ursolic acid from the standard curve. Detection of the pure chemicals of oleanolic acid and ursolic acid from the spike samples as well as in the samples was determined by using the analytical software called MassLynx, Version 4.0 (Micromass 2002). The chromatograms of oleanolic acid and ursolic acid in standard solution and in the sample are shown in Figure 7.2 (i and ii).

7.2.7 Recovery

To test the extraction recovery, to the 1.0g dried plant seed powder 50 µL of the standard solution (made up of oleanolic acid and ursolic acid, concentration of 12.0mg/l), was added, before and extraction was done

through the same procedure as described above (7.2.5). The amount of solvent used for a single extraction was 40ml (which had been concentrated 10 times), so a spiked sample extractant has a concentration of the stocked solution 0.15ppm (or mg/L). Each final extractant dried for analysis was designated as either spiked or non-spiked (e.g. extract from Moringa powder spiked with pure chemical was designated as Spiked Moringa, MS and vice versa- Moringa Non-Spiked, MNS).

Therefore, for every sample there were two sets of solutions, spiked (S) and non-spiked (NS). Out of these, UPLC-MS analysis was conducted and data evaluated using the MassLynx Version 4.0 software (Micromass, 2002). UPLC-MS analyses were accomplished as detailed above.

The recovery of oleanolic acid and ursolic was determined as follows

$$\text{Recovery (\%)} = \frac{(A - B)}{C} \times 100\%$$

Where, A is the result after addition, B is the amount of sample without adding standards, C is the added amount of the standards.

Recovery was studied in three replicates of the samples (both spiked and non-spiked), and the average or mean values ± SD were recorded (i.e. standard deviation, as recovery or relative standard deviation, RSD). The results are presented as a chromatogram and in tables.

7.2.8 Quantification of Oleanolic Acid and Linearity in Moringa Seed Powder

Quantification

The content was computed from the relation below.

$$\text{Amount of Chemical in Sample (µg/g)} = \frac{\text{C (ppm)}}{\text{(1g)}} \times \frac{\text{(40ml solvent)}}{\text{(10 x concentration)}}$$

Where C is concentration of injected sample (non-spiked)

Linearity

A calibration standard of 0.0012, 0.0024, 0.012, 0.06, 0.12, 0.06 mg/mL were prepared for oleanolic acid from the stock solution by the serial dilution of methanol and corresponding peak area from the chromatograph generated. Using Microsoft Excel (Office 2010), these values were used to construct calibration curve in the range of 0.0012–0.6 (ppm or mg/ mL) for oleanolic acid. A regression curve was used to calibrate the linearity relationship between the peak area of the chromatograph and concentration of oleanolic acid estimation. The calibration curve was prepared by determining the best fit of the peak area ratio against concentration (Devaraj *et al.*, 2011).

7.3. Results

7.3.1 Separation of Oleanolic acid and Ursolic acid in Standard solution

Oleanolic acid and ursolic acid mixed in the standard solution could be successfully separated using the mobile phase 10mM ammonium acetate as solvent A and 100% methanol as solvent B at a ratio of 25:75 (v/v) from the standard solution. The chromatograph of oleanolic acid and ursolic acid is shown in Figure 7.1, and the separation of the two compounds was approaching the baseline (Wang *et al.*, 2008).

Retention time for oleanolic acid and ursolic acid was 7.0min and 7.4min respectively. Both compounds were detected as a quasi-molecular ion peak at *m/z* 455 $[M-H]^-$, with oleanolic acid being represented by the higher peak, and ursolic acid the slower peak.

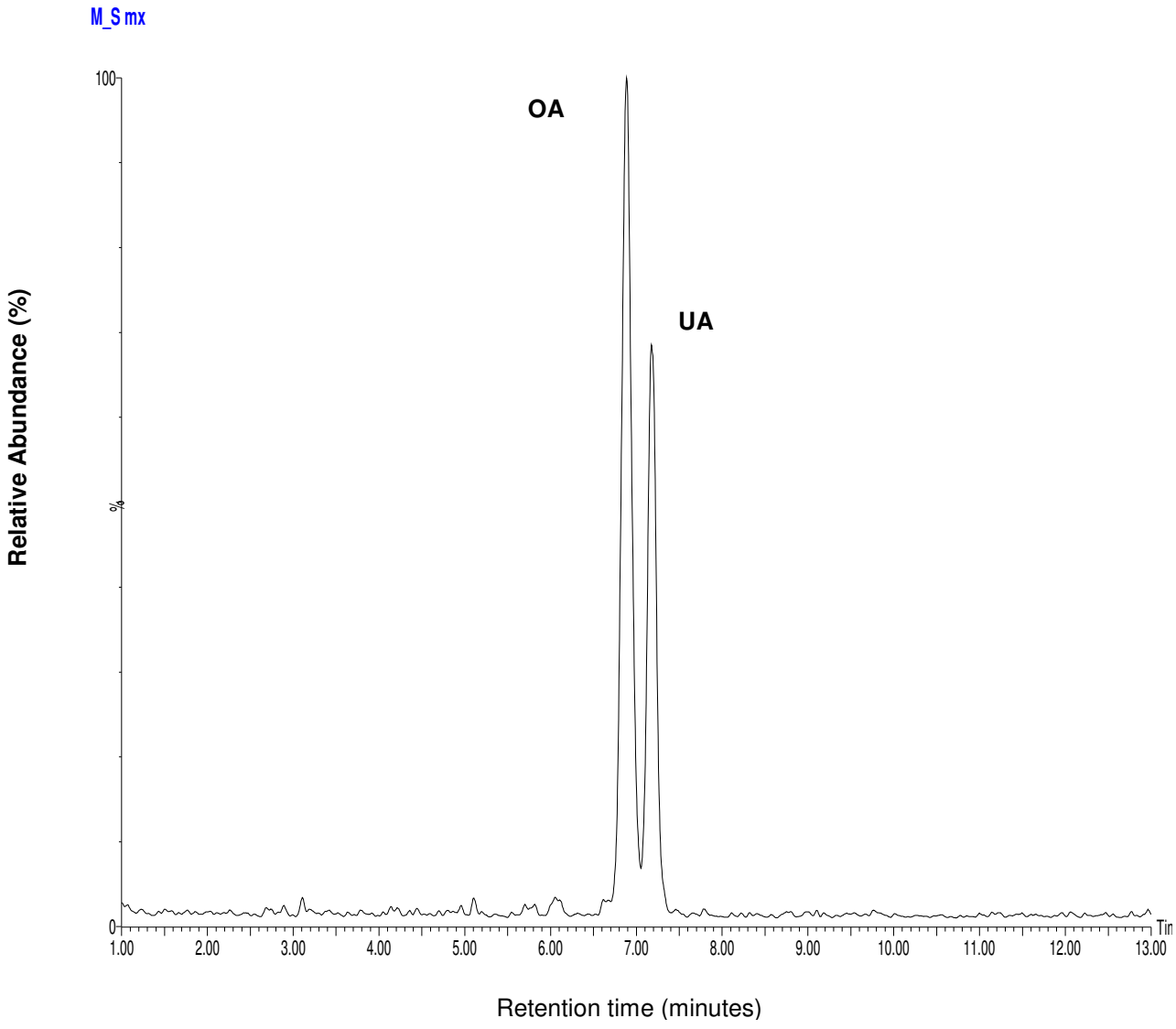

Figure 7.1 Chromatogram of oleanolic acid and ursolic acid in standard solution OA, oleanolic acid; UA, ursolic acid

7.3.2 Recovery of Oleanolic acid and Ursolic acid in the Spike Specimens

Recovery of oleanolic acid and ursolic acid in the spiked samples was obtained in both the Moringa and Pawpaw seed powder but not in the fish tissues. The recovery of oleanolic acid and ursolic acid in the spiked Moringa seed sample is presented in Tables 7.1 and 2.2. Mean recovery of oleanolic acid and ursolic acid in Moringa seed was 96.467% (n=3); %RSD = 9.8 and 90.3% (n=3) %RSD = 5.7 respectively.

Table 7.1 UPLC Instrument detection of concentration for Oleanolic Acid Recovery based on Spiked (0.15mg/L of standard solution) and Non-Spiked Samples of Moringa Seed Powder.

Quantity Injected (µg/l)	Non Spikes (NS ppm)	Spikes (S+0.15 ppm)	% Recovery µg/g	% RSD
0.124	0.133	0.272	92.600	
0.119	0.129	0.264	89.600	
0.127	0.118	0.279	107.200	
		Mean (± s.d) (n=3)	96.467 ± 9.416	9.760

Table 7.2 UPLC Instrument detection of concentration for Ursolic Acid Recovery based on Spiked (0.15mg/L of standard solution) and Non-Spiked Samples of Moringa Seed Powder.

Quantity Injected (µg/l)	Non Spikes (NS ppm)	Spikes (S+0.15 ppm)	% Recovery µg/g	% RSD
0.123	-	0.143	95.500	
0.127	-	0.128	90.200	
0.124	-	0.135	85.200	
		Mean (±s.d) (n=3)	90.300±5.151	5.704

7.3.3 Detection and Quantification of Oleanolic Acid in Moringa Seed

From the UPLC analysis only oleanolic acid was detected in the non-spiked Moringa seed powder (MNS) but not in the other non - spiked samples (i.e. Pawpaw seed or fish tissues). UA was not detected or found in any of the non-spiked samples (i.e. Pawpaw seed, Moringa seed or fish tissues). Results obtained are shown Table 7.3. The average content of oleanolic acid in Moringa Seed Powder was 0.508µg/g ±0.032. The chromatograph of oleanolic acid from the Moringa seed is shown in Figure 7.2.

Table 7.3 UPLC Instrument detection of concentration Oleanolic Acid in Moringa Seed Powder based on Non-Spiked Samples

Quantity Injected (µg/l)	Non Spikes (NS ppm)	Recovery µg/g
0.127	0.118	0.472
0.124	0.133	0.533
0.119	0.129	0.517
	Average (n=3)	0.508 ± 0.032

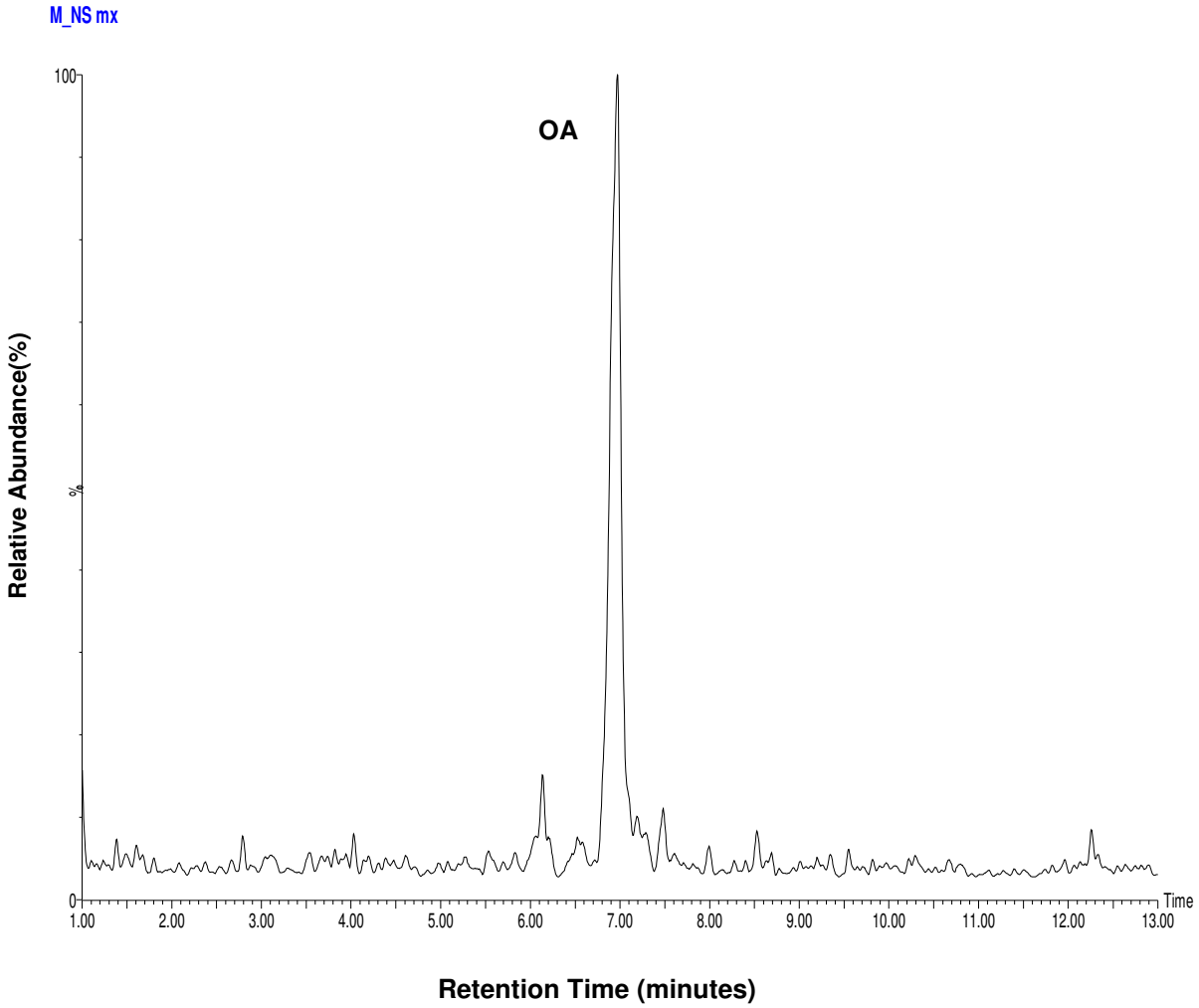

Figure 7.2 Chromatograph confirming the presence of oleanolic acid in Moringa seed non-spiked

7.3.4 Linearity of Oleanolic acid to Detection in Moringa Seed

The relationship between the estimated amount and detection is presented in Table 7.4 and Figure 7.3. The calculated regression equations between the peak area (y) against concentration (x) and their coefficients of determination (R^2) were as follows: oleanolic acid, y= -1074.5 x^2 + 5760.9 x + 3.5 and (R^2 = 0.9993).

Table 7.3 TargetLynx Calibrated concentrations of oleanolic acid expected in Moringa Seed

Concentration(ppm), (x)	Detection Peak area of OA (y)
0.0012	12.1
0.0024	19.3
0.012	92.9
0.06	402.1
0.12	644.8
0.60	3074.0

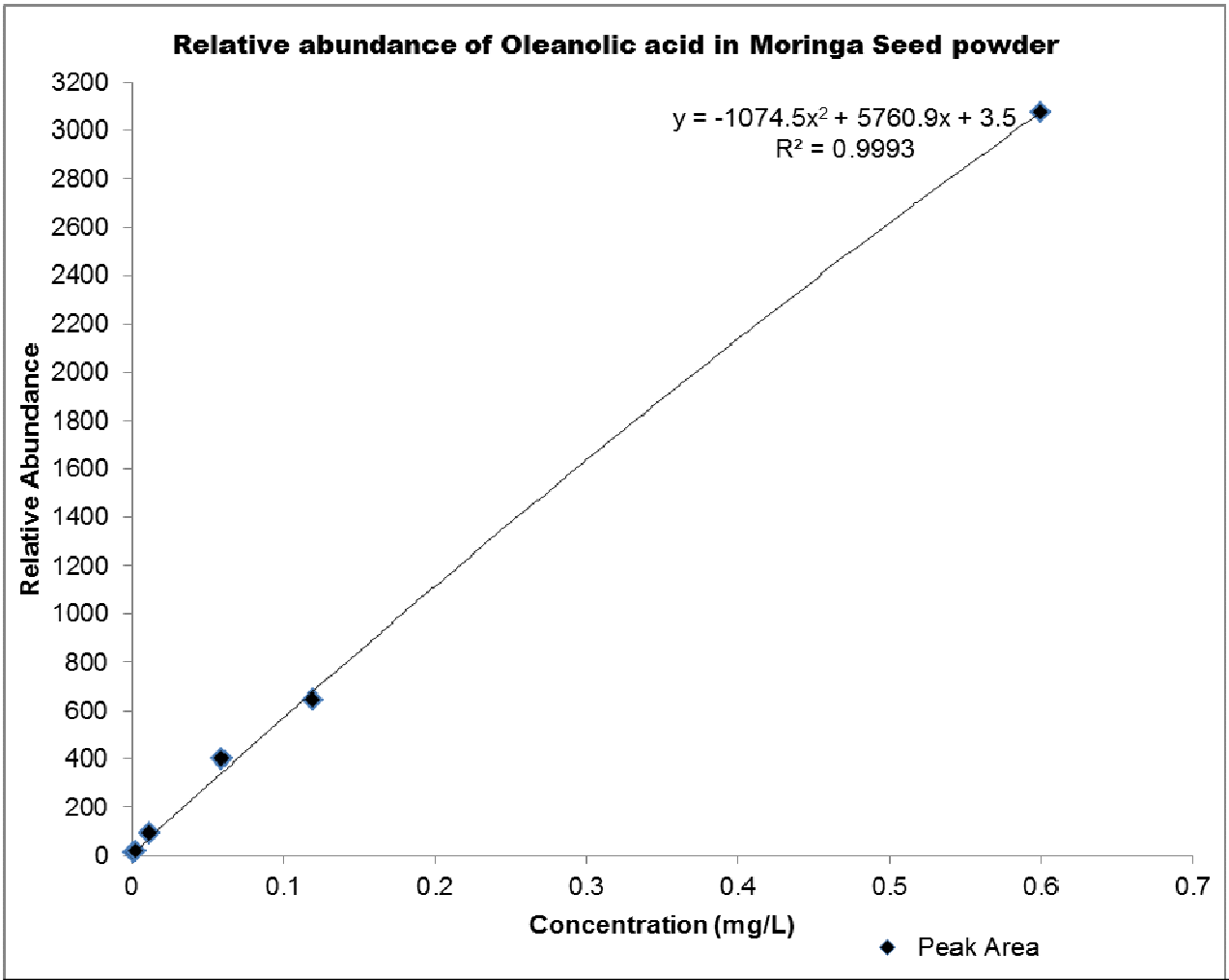

Figure 7.3 Regression between the chromatographic peak area (y) and Olieanolic Acid concentration (x) in Moringa seed. (Y = -1074.5 x^2 + 5760.9 x + 3.5 and R^2 = 0.9993)

7.4 Discussion

7.4.1 Recovery

Oleanolic acid and ursolic acid are isomeric triterpenic acids with similar chemical structure, that commonly existing in the same plant. Therefore, it is very difficult to separate them when determining a complex mixture with high performance liquid chromatography (HPLC). However, an ultra-performance liquid chromatography (UPLC), which runs on chromatographic principles designed to have a column packed with very small particles with higher flow rates for increased speed, with superior sensitivity and high resolution enables rapid separation and quantification (Swartz, 2005; Zhang *et al.*, 2012). Using an UPLC-ESI-MS/MS, oleanolic acid and ursolic acid could be recovered and separated when added to a plant extract. In this study, the mean recovery (MR) of oleanolic acid was 96.5% (n=3) with relative standard deviations (%RSD) of 9.8, whiles that of ursolic acid was 90.3% (n=3) and %RSD was 5.7 was achieved. The limit of detection (LOD) was similar at 0.0006ppm (0.6ppb).

Studies have shown that several factors determine the effective recovery of a compound of a complex mixture and such factors may include the affinity of the compound for the solvent, the solvent to sample volume ratio, and type and concentration of extracting solvent (Liang *et al.*, 2009; Tiwari *et al.* 2011; Xia *et al.*, 2012; Xu *et al..*, 2012).The recovery of the spiked oleanolic acid and ursolic acid from the samples show a clear affinity between the solvents used and the respective compounds, with ≥90% recovery.

7.4.2 Separation of Oleanolic Acid and Ursolic Acid in Standard Solution

Oleanolic acid and ursolic acids were conveniently separated using the mobile phase 10mM ammonium acetate as solution [A] and 100% methanol as solution [B] in a ratio of 25:75, (v/v), from the standard solution using UPLC-ESI-MS/MS, on retention time of 7.0min, and 7.4min for O LEANOLIC ACID and ursolic acid respectively.

Several forms of HPLC methods such as reversed-phase high performance liquid chromatography (RP-HPLC) and HPLC in tandem with mass spectrometry (HPLC- MS/MS) have been used to separate oleanolic acid and ursolic acid in complex mixtures found in medicinal plants and herbs. However, the retention time tends to be long. For instance using RP-HPLC, Wang *et al.* (2008) reported a retention time between 10 and 20 minutes for oleanolic acid and UA respectively. In the study of Xu *et al.*, (2012), retention time for oleanolic acid was 20.58 minutes, whiles that reported for UA was 21.57 minutes. Liang *et al.*, (2009) used HPLC, with a mobile phase made up of methanol-0.2% ammonium acetate in water (83:17), to completely separate oleanolic acid and ursolic acid from the herb O*ldenlandia diffusa,* at a retention time between 25 and 30. In all of these studies oleanolic acid appeared earlier on the chromatogram than the ursolic acid. In this study the retention time for oleanolic acid was 7.0 minutes, whiles that for ursolic acid was 7.4 minutes. Thus, from the chromatograph (Figure 7.1), in this study, oleanolic acid is the one with the higher peak, and ursolic acid the shortest. This therefore indicates that oleanolic acid and ursolic acid can be separated.

Using the mobile phase 10mM ammonium acetate as A and 100% methanol as B in a ratio of (25:75, v/v), a complete separation of oleanolic acid and ursolic acid was successfully achieved (Figure 7.1), from the standard solution. Liang *et al.* (2009) satisfactorily obtained a chromatographic separation of oleanolilc acid and ursolic acid using a mobile phase of methanol-0.2% ammonium acetate in water (83:17), confirming that the mobile phase selected was adequate to separate oleanolic acid and ursolic acid.

Oleanolic acid and ursolic acid are isomeric compounds with similar chemical structure and therefore difficult to separate. Makarov *et al.* (2009) reported that using, UPLHC with an ACQUITY BEH C18 (1.7µm, 2.1 x 100mm) columns with isocratic mobile phase, at temperature of 45^{o}C, they could separate 3 structurally related nitramine compounds. Similarly, Zhang *et al.*, (2012) used ultra-high performance liquid chromatography / quadrupole time-of-flight mass spectrometry (UHPLC/QTOF-MS) with an ACQUITY BEH C18 (1.7µm, 2.1 x 100mm) columns with isocratic mobile phase to identify flavonols and completely separate complex triterpene glycosides in *Luo-Han-Guo* extract, using a mobile phase consisting of (A) 0.1% formic acid in water and (B) acetonitrile. The authors also stated that triterpene glucosides could be fully ionized in negative ESI mode, thus enhancing their separation. This indicates that compounds with similar chemical structures that could be ionized may be separated. The separation column for this study was Waters ACQUITY BEH C18 (1.7µm, 2.1 x 100mm), with isocratic mobile phase which could separate the two isomeric triterpene acids. Thus, under the right conditions UPLC-ESI-MS/MS is capable of separating isometric compounds like oleanolic acid and ursolic acid, and be clearly identified.

7.4.3 Detection of Oleanolic Acid and Ursolic Acid in the Specimens

According to Tiwari *et al.* (2011) the type of solvent and extraction procedure influences the successful determination of biologically active compounds from plant materials. Some of the merits of a proper plant solvent include the ease of evaporation at low heat, and promotion of rapid physiological absorption of the extract. Xia *et al.* (2012) found that maximum extraction of oleanolic acid and ursolic acid could be achieved from plants using a mixed solvent of 95% ethanol and 5% water at a liquid to solid ratio of 20:1.

In this study a mixture of 50% acetonitrile, 45 % methanol and 1% chloroform, as extracting solvent was used in liquid to solid ratio of 20:1(v/v) The use of combinations of solvents conforms to studies by Liang *et al.* (2009), Tiwari *et al.* (2011), Xia *et al.* (2012) and Xu *et al.* (2012). The triterpenic acids, oleanolic acid and ursolic acid often exist simultaneously in the same plants. From three sets of samples of all the specimen (extracts of Moringa, Pawpaw seed powder, Fish tissues) were subjected to the same test described above. The chromatogram (Figure 7.1 & 7.2) obtained were used to identify oleanolic acid and ursolic acid using the retention times and peak height in the two figures. In Figure 1, two peaks were observed with oleanolic acid at 7.0 minutes, whiles ursolic acid was 7.4 minutes. In Figure 7.2 only one peak was observed which came out of the Moringa extract. By comparison of retention time and chromatographic peak height, with that of the standard it was authenticated that oleanolic acid was present in Moringa seed powder, but not in the other specimen (i. e. Pawpaw seed, fish tissues). Thus the presence of oleanolic acid could only be detected in the Moringa seed powder.

According to Swartz (2005), UPLC technology is an improved form of HPLC, to provide increased resolution, speed and sensitivity to accurately detect micro-contents of complex matrix efficiently. In this study only oleanolic acid was detected in the non-spike Moringa seed with a clear distinct chromatographic peak (Figure 7.2). Ursolic acid could not give a distinctive chromatographic peak as seen in Figure 7.2. The over 90% of recovery for oleanolic acid and ursolic acid from the spiked samples, clearly demonstrates the efficiency of the extraction procedure and solvent. Several investigators such as Liang *et al.* (2009); Tiwari *et al.* (2011); Xia *et al.* (2012) and Xu *et al.* (2012) have used UPLC to successfully separate and quantify oleanolic acid and ursolic acid in plant samples.

The chromatograph in Figure 7.2 showed a wave at the base which demonstrates the presence of some bioactive compounds but in small quantities. Non detection of UA in Moringa could mean that it was present but in small quantity. Pollier and Goosens (2012) have reported that oleanolic acid occurs both as a free acid and as an aglycone precursor for triterpenoid saponins in which it is linked to one or more sugar chains in plants. It also accumulates at very low concentrations in plants, this could explain the reason why both oleanolic acid and ursolic acid were not detected in Pawpaw. The absence of oleanolic acid and ursolic acid in Pawpaw seed powder could also mean they were present in their derivatives such as oleanolic glycoside (Das, 1980), oleanolic acid-3 β-glucoside and oleanolic acid 3-β-D-glucuronide (Das *et al*., 2011).

7.4.4 Quantification of Oleanolic Acid and Linearity in Moringa Seed Powder

The average content of Oleanolic acid in Moringa Seed Powder obtained was 0.5079µg/g ±0.032, (n=3), with a correlation coefficient of linear regression analysis (i.e. coefficient of determination, r^2) of 0.9993. It has been established that the Moringa plant parts contain pyhtoestrogens including β-sitosterol, caffeoylquinic acid, quercetin and kaempferol (Anwar *et al*., 2007; Amaglo *et al.,* 2010; Devaraj *et al*., 2011); saponin in the seed powder (Kawo *et al*., 2009); steroids, triterpenoids, flavonoids, saponins, and anthraquinones in the leaves (Kasole *et al.,* 2010); steroids, triterponoids, saponins in the bark (Lambole and Kumar (2011), and saponins, flavonoids and steroids in the leaves (Bamishaiye *et al*., 2011).

7.5 Conclusion

The extraction method adopted followed standard procedure, however, ursolic acid could not be quantified in Moringa. As to why both oleanolic acid and ursolic acid could not be recovered in Pawpaw seed can also not be explained. The plausible explanation is that triterpines usually are present in plants in micounits and also exist in their glycoside forms. New extraction procedures need to be explored in future studies, since establishing their existence is critical to their usage. It could be concluded from available literature that the findings from this study is the first report of the identification of oleanolic acid in Moringa seeds indicating that oleanolic acid is present in Moringa seeds. The presence of oleanolic acid in Moringa seeds adds more credence to its potential use as antifertility agent, which could be exploited in the control of tilapia precocious breeding. Further studies need to be conducted on Moringa seed from other parts of Sub-Saharan Africa (other than Zambia) to find out the extent of oleanolic acid and ursolic acid composition.

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Chapter 8

Conclusions and recommendations

Tilapia production plays an important role in global food security, in particular amongst the rural communities of developing countries. The viability of commercial and small-scale Mozambique tilapia (*Oreochromis mossambicus*) culture is complicated by an early onset of sexual maturity, which results in uncontrolled reproduction, overpopulation and retarded growth in aquaculture systems. Various techniques have been developed to control the precocious breeding pattern of tilapia in culture systems, but they all have drawbacks. For example the most effective techniques (hormonal sex reversal) is not suitable for use by rural small scale farmers due to its complexity and limited access to the required resources and substances, e.g. steroid hormones and alcohol.

Natural reproduction inhibitors (phytochemicals) found in traditional medicinal plants such as Pawpaw (*Carica papaya*) and Moringa (*Moringa oleifera*), provide potential alternative for the control of sexual differentiation in fish. Previous studies have indicated that phytochemicals that behave like natural steroid-like substances, particularly with an estrogen-like biological activity, occur in these plants. Such phytochemicals are referred to as phytoestrogens, and can be defined as substances or metabolites that induce biological responses in vertebrates by mimicking or modulating the actions of endogenous oestrogens (such as 17β-estradiol), usually by binding to estrogen receptors.

The hypothesis was set that a diet containing natural phytogenic compounds will affect the reproductive potential of Mozambique tilapia (*Oreochromis mossambicus*; MT) in aquaculture systems. The study therefore investigated the potential of Pawpaw seed meal (P) and Moringa seed meal (M), as part of a commercial tilapia diet, to be used as endocrine disrupting compounds (EDC's) to control the reproduction of sexually mature Mozambique tilapia (MT; 20-45g), to inhibit the attainment of sexual maturity in immature MT (2-8g), and to influence sexual differentiation of MT fry (9-12 days post-hatch) to produce all-male populations.

The effect of PSM and MSM as endocrine disrupting compounds to control reproduction in sexually mature Mozambique tilapia (20-45g)

This first part of the study investigated the effect of PSM and MSM included at levels of 0.5, 1.0, 5.0, 10.0 and 15.0g/kg of a commercial tilapia feed (BD), on the gonad integrity and function of sexually mature Mozambique tilapia. A total of 550 fish were allowed to acclimatize for 7 days, and were then fed the respective treatment diets for a period of 60 days. Histological examination of gonad tissue indicated that diets containing levels of 5.0, 10.0, & 15.0 g/kg BD of Pawpaw and Moringa respectively, severely affected gonad integrity. The most pronounced effects observed in the male fish included degeneration of the seminiferous tubules, disintegration of the spermatozoa, and the presence of testes-ova. In the female fish, atretic oocytes and depletion of yolk particles were observed.

The synergistic effect of PSM and MSM to control reproduction in sexually mature Mozambique tilapia (20-45g)

This part of the study investigated the potential interaction of the phytochemicals present in the Pawpaw and Moringa seeds in affecting gonad integrity and function of sexually mature Mozambique tilapia. A total of 150 fish were allowed to acclimatize for 7 days, and were then fed the respective treatment diets for a period of 60 days. The combination of Pawpaw and Moringa did not prove to have a more pronounced effect on gonad integrity than what was observed when the individual experimental compounds were included in the respective experimental diets.

The effect of PSM and MSM as endocrine disrupting compounds to inhibit the attainment of sexual maturity in sexually immature Mozambique tilapia (2-8g)

The second part of the study investigated the potential of Pawpaw and Moringa included at levels of 0.5, 1.0, 2.0, 5.0, 10.0g/kg of a commercial tilapia feed (BD), to inhibit sexually immature Mozambique tilapia te attain sexual maturity. A total of 1650 fish were acclimatized for 7 days, and were then fed the respective treatment diets for a period of 60 days. Eggs were observed in the ovaries of the sexually immature fish, but spawning did not occur. Testis integrity and function was also affected, and degeneration of the gonad tissue was observed. Ova-testes were observed in fish that received 10.0g of Pawpaw and/or Moringa/kg basal diet. Both Pawpaw and Moringa also were effective in skewing the sex ratio to yield 71% male and 29% female fish respectively.

The effect of PSM and MSM as endocrine disrupting compounds to influence sexual differentiation in Mozambique tilapia fry (9-12 days post-hatch)

This component of the study investigated the effect of Pawpaw and Moringa included at a levels of 15g/kg of a commercial tilapia feed (basal diet, BD), on the sexual differentiation of Mozambique tilapia fry. A total of 2400 fish were acclimatized for 7 days, and were then fed the respective treatment diets for a period of 60 days.

The inclusion of the crude form of Pawpaw and Moringa seeds believed to contain phytogenic chemicals such as genistein, quercetin, kaempferol, diadzein and, β-sisterol, resulted in significant degree of masculinization (up to 65%) in a mixed sex population of Mozambique tilapia fry. It can thus be concluded that the sexual differentiation of the fry could be successfully manipulated to obtain populations with a predominant male gender.

Biochemical analysis of the phytogenic components of Pawpaw and Moringa

The standard method of extraction allowed for the isolation of only oleanolic acid, but not ursolic acid in Moringa. The reason for the isolation of oleanolic acid and ursolic acid could not be recovered in Pawpaw seed can also not be explained. The plausible explanation is the triterpenes usually are present in plants in microunits and also exist in their glycoside forms, which can make extraction difficult. New extraction

procedure needs to be explored in future studies, since establishing their existence is critical to their usage. It could be concluded from available literature that the findings from this study are the first report of the identification of oleanolic acid in Moringa seeds. This study indicated that oleanolic acid is present in Moringa seed. The presence of oleanolic acid in Moringa seeds add more credence to its viability as antifertility agent, hence could be exploited in the control of tilapia precocious breeding. Further studies need to be conducted on Moringa seed from other parts of Sub-Saharan Africa (other than Zambia) to find out the extent of oleanolic acid and ursolic acid composition. This needs to be investigated to ensure that the availability of the plant seeds is not a limiting factor for its potential use to control tilapia breeding.

Summary

Pawpaw and Moringa seeds can be considered as a possible alternative to induce sex reversal in mixed sex tilapia populations. This could be of particular importance to aquaculture development in rural areas of Sub-Saharan African countries, given the abundant year round availability of these compounds. The study indicated that both Pawpaw and Moringa seeds contain bio-active chemicals that are capable of disrupting the gonad function, differentiation and sexual maturation of Mozambique tilapia. Oleanolic acid was successfully isolated in Moringa seeds, and this study is the first report on the successful isolation in Moringa seeds. It has also been proven to have antifertility effects in vertebrates.

It was proved that both Pawpaw and Moringa seeds have antifertility properties that can be exploited to control or prevent reproduction of Mozambique tilapia in aquaculture systems. Sperm production was affected, as evident in the degeneration of the testicular tissue samples. Egg production, ovulation and spawning were all affected, as evident in the difference in colour of the degrading eggs, as well as the absence of spawning. Ovo-testes were observed in cases where diets containing 10.0g Pawpaw and/or Moringa/kg basal diet were fed.

Further studies are required to optimise the preparation of the experimental compounds; to determine the optimal age at which to commence administration of the experimental compounds; to determine the optimal duration of administration; to determine the optimal inclusion levels and potential interaction with environmental factors such as water temperature and pH.

Further studies are, however, required to understand the exact mechanism through which the Pawpaw and Moringa seeds exert their effect. It is possible that the results observed in the present study can be ascribed to the action of not just one but multiple phytogenic compounds present in both the Pawpaw and Moringa seeds. The biochemical composition of Pawpaw and Moringa seeds needs to be studied to determine the different phytogenic compounds present in these seeds, as well as the relative concentrations with regards to each other.

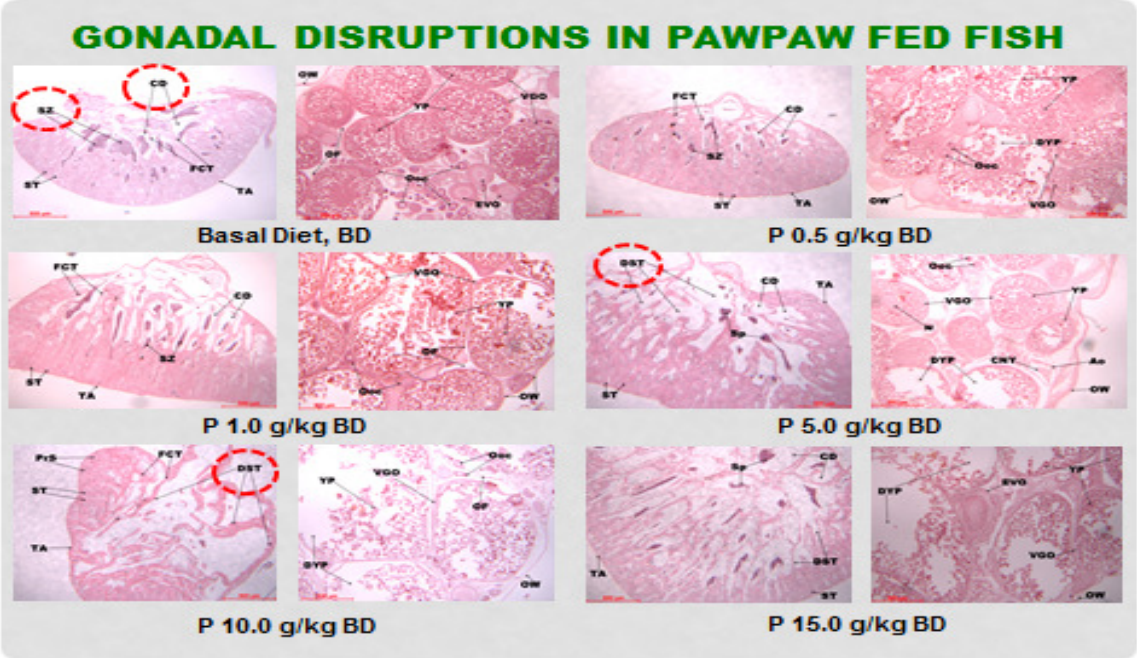
Additional studies need to determine the optimal inclusion levels of the phytogenic compounds, as well as how their efficacy to manipulate the reproductive potential and ability of Mozambique tilapia are influenced by environmental factors such as water temperature.

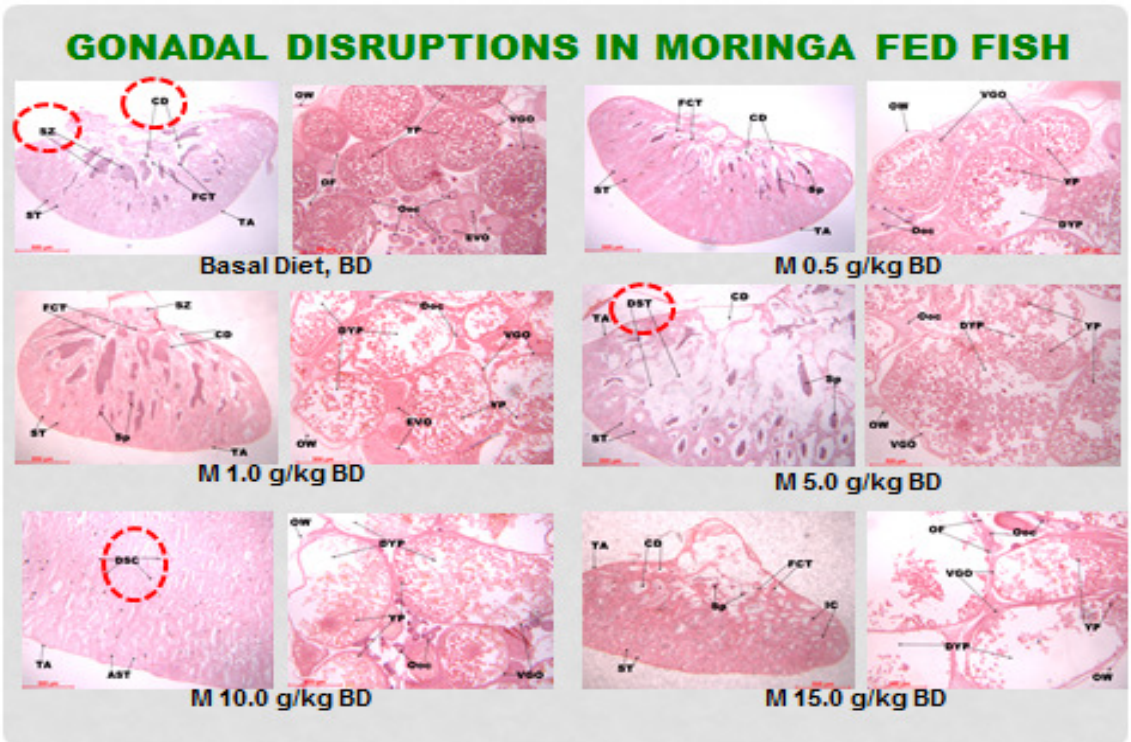
Eggs were observed in the ovaries of sexually immature fish, but spawning did not occur. Future studies should investigate the influence of the EDCs present in Pawpaw and Moringa seeds on egg formation, maturation and eventual release. The potential interaction of the phytochemicals present in the Pawpaw and Moringa seeds with environmental factors to influence egg production and ovulation, and sperm production also warrant further investigation.

APPENDICES

A] Pictorial information of gonadal integrity (in sexually mature and immature Mozambique tilapia, *Oreochromis mossambicus,* fed Pawpaw and Moringa seed powder meal and different Inclusion levels)

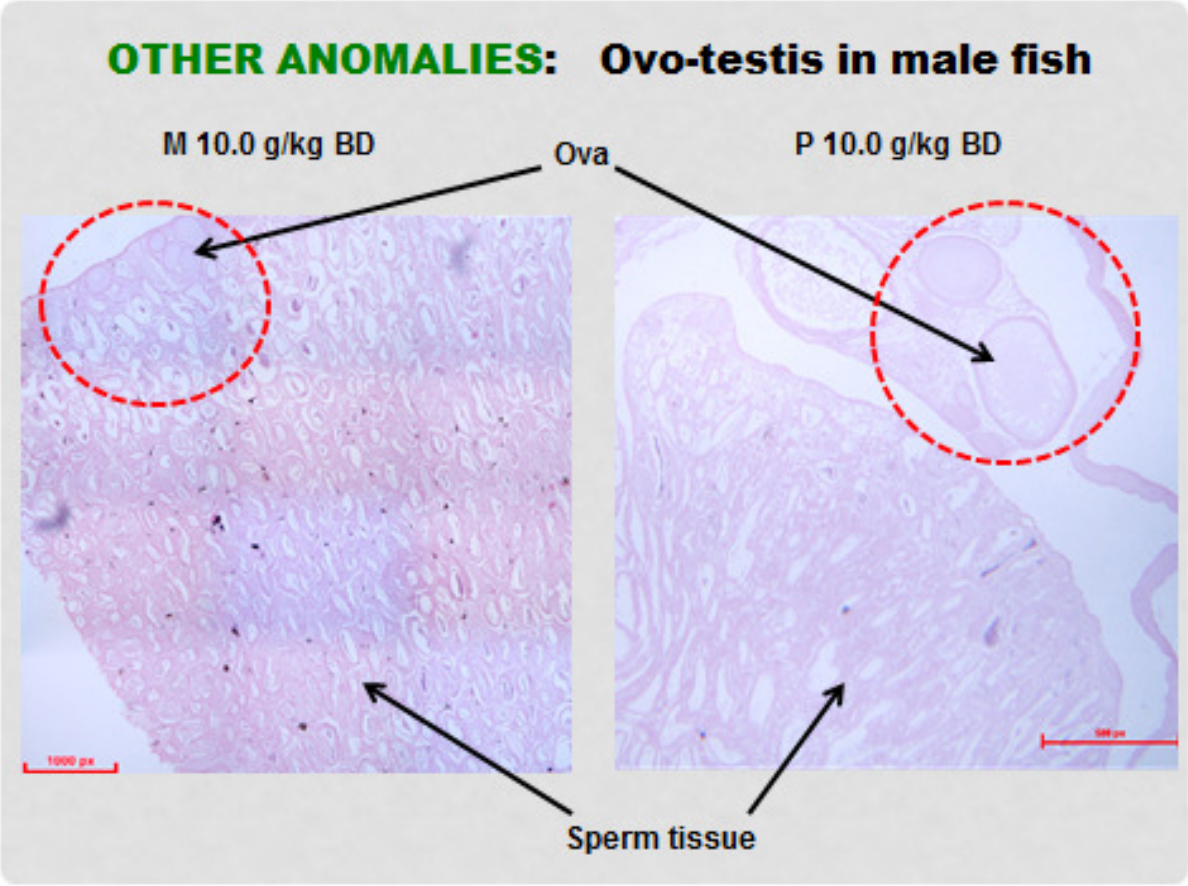
(i) Gonad integrity in sexually mature *Oreochromis mossambicus*



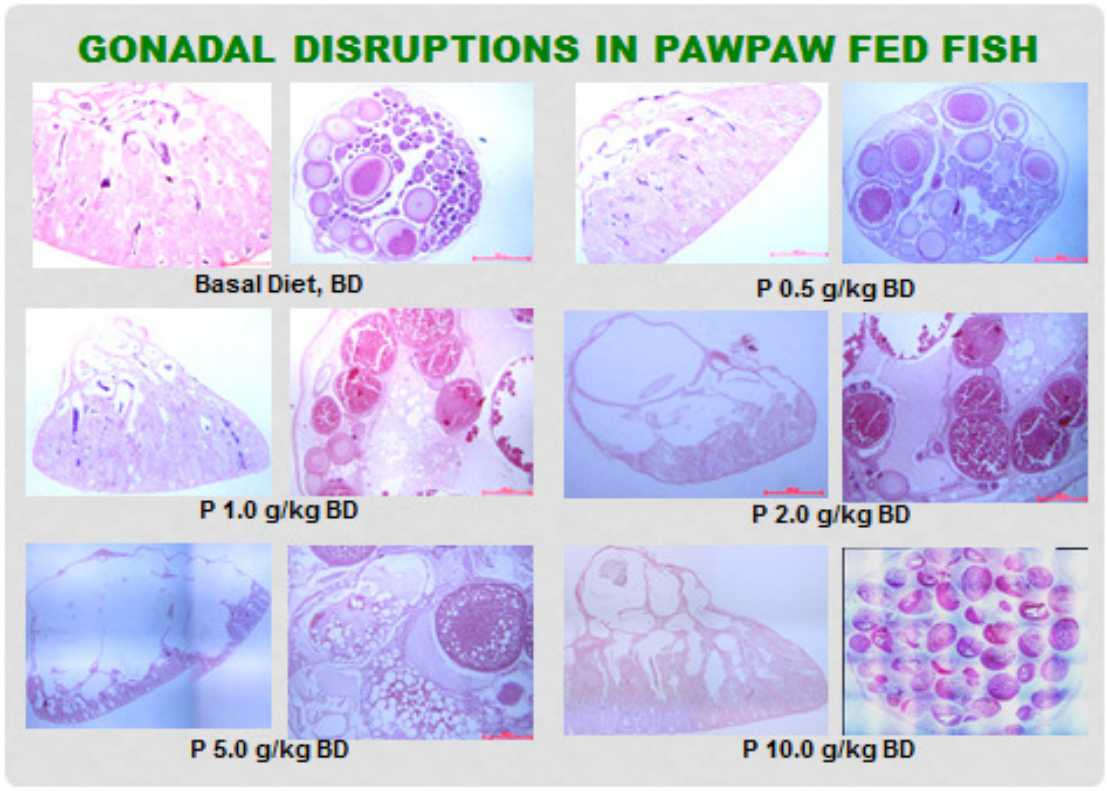


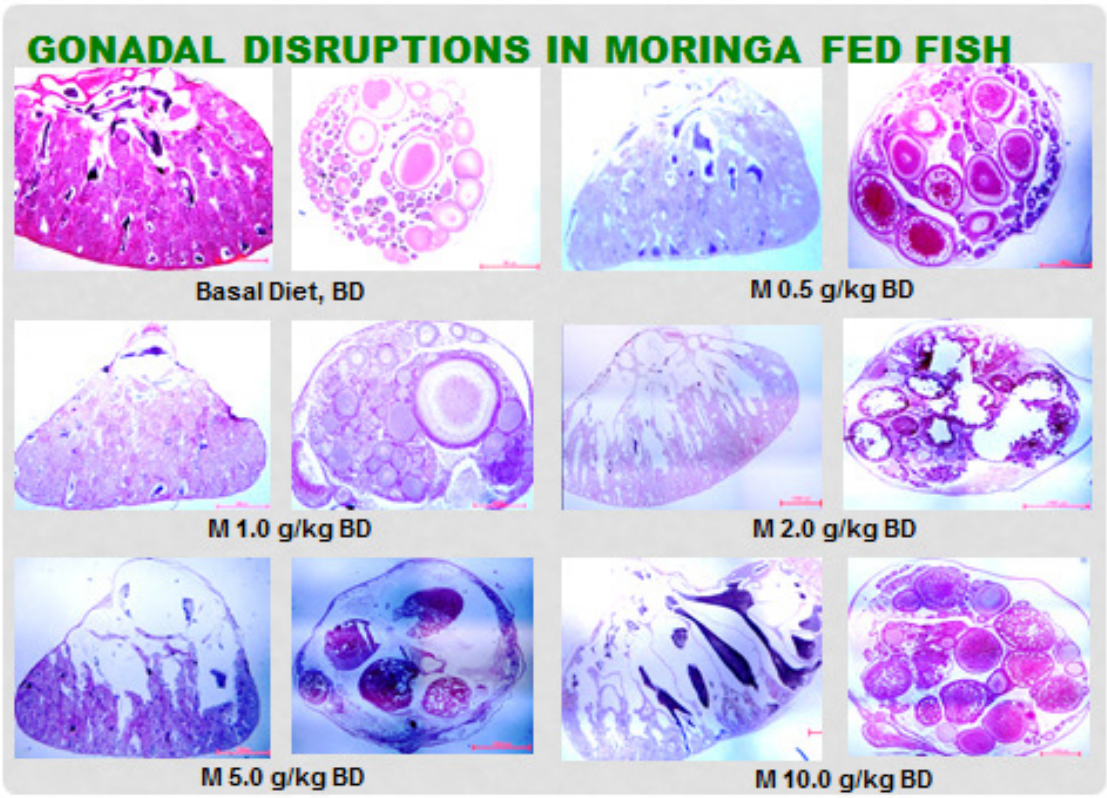
Key

AST = atrophied seminiferous tubules;
CD = collecting duct**;**
DSC = disintegrating sperm cells;
DST = degenerating seminiferous tubules;
DYP = depleting yolk particles;
EVO = early developing oocytes;
FCT = fibrous connective tissue;
OF = ovigerous follicle;
Ooc = developing oocytes;
OW = ovarian wall;
PrS = Primary spermatogonia;
Sp = spermatids;
ST = seminiferous tubules;
SZ = spermatozoa;
TA = tunica albuginea;
VGO = vitellogenic oocytes;
YP = yolk particles.
Bar = 500µm



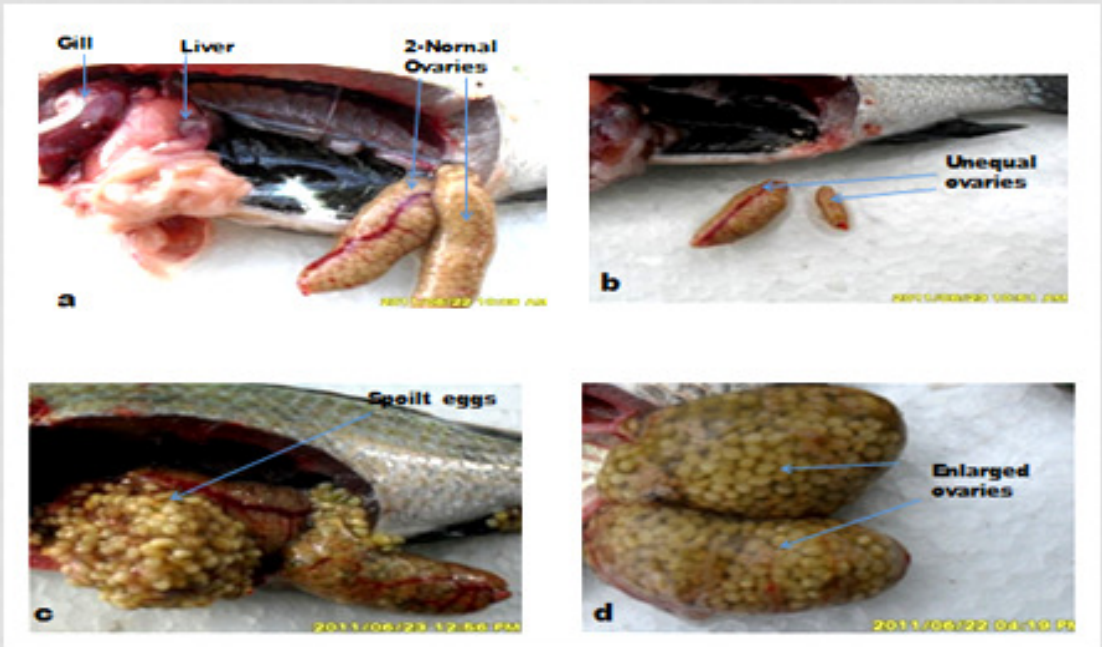
(ii) Gonad integrity in sexually immature *Oreochromis mossambicus*





OTHER ANOMALIES: Ovo-testis in male fish

INFLUENCE OF PAWPAW AND MORINGA ON GONAD DEVELOPMENT



a) Normal ovaries, similar in size and shape, b) unequal sized gonads, c) enlarged ovaries with degenerated or deformed eggs (abnormal colour), and d) enlarged ovaries

B] List of Chemicals used in the experiment

1. 17-α methyltestosterone
2. Alcohol (70-100%, absolute)
3. Bouin's solution (acetic acid 5%, formaldehyde 9%, picric acid 0.9%)
4. DPX mountant (a mixture of distyrene, plasticizer, and xylene)
5. Purified forms of oleanolic acid and ursolic acid
6. Ethanol
7. Methanol
8. Acetonitrile
9. Chloroform
10. Dichloromethane
11. Formic acid
12. Ammonium acetate

C] Histological preparation of slides (as used in the Laboratory of Histology Department, Faculty of Health Sciences, Tygerberg Campus, Stellenbosch University)

i) Tissue processing, using Shanddon Elliot Duplex Processor

The tissue passes through the following chemicals for 17 24 hours

1. 10% Formalin	(7-10 hours)
2. 70% Alcohol	(2 hours)
3. 96% Alcohol	(1.5 hours)
4. 96% Alcohol	(1.5 hours)
5. 99% Alcohol	(1 hour)
6. Absolute Alcohol	(1 hour)
7. Absolute Alcohol	(1 hour)
8. Absolute Alcohol	(1 hour)
9. Xylene	(1.5 hours)
10. Xylene	(1 hour)
11. Wax I	(1 hour)
12. Wax II	(1 hour)

ii) Embedding (instrument: LEICA EG 1160)

The tissues were embedded in paraffin wax at 60 ^{o}C, in an embedding instrument

iii) Sectioning cutting (instrument Microtome, Model, Bright 5040)

Using a microtome, 5µm -8µm thickness sections were cut.

All tissues were oriented on the wax block in such a way to produce transverse sections (TS).

However, few tissues of the testis were cut in longitudinal sections due to the size of the organ.

For each specimen at least 3 sections were placed on a glass slide in warm water.

iv) Staining procedure (LEICA AUTO STAINER, XL)

All the slides were stained for 25 minutes using with Ehrlich's haematoxylin and counterstained with eosin, through the following steps: Haemtoxylin and Eosin (H&E) staining procedure

STEP 1 **Incubation**

a) Incubate the slides for (at least 60 minutes) in an autoclave with a constant temperature of 60 ^{o}C

STEP 2 **Hydration**

a) Submerged the slides in Xylene-solution for 2 minutes (repeat 2x)

b) Submerged the slides in pure alcohol (100%) for 1 minute (repeat 2x)

c) Submerged the slides 96% alcohol for 1 minute (repeat 2x)

d) Submerged the slides 70% alcohol for 1 minute (repeat 2x)

e) Wash with distilled water (dH_2O) for 2 minutes

STEP 3 **Staining**

a) For 4 minutes, stain with Haematoxylin

b) Wash for 3 minutes with distilled water (dH_2O)

c) For 2.30 seconds, stain with Eosin

d) Wash for 2 minutes with distilled water (dH_2O)

STEP 4 **Dehydration**

a) Submerged the slides in 70% alcohol for 0.20 seconds

b) Submerged the slides in 96% alcohol for 0.15 seconds (repeat 2x

c) Submerged the slides pure alcohol (100%) for 0.15 seconds (repeat 2x

d) Submerged the slides Xylene-solution for 0.30 seconds (repeat 2x

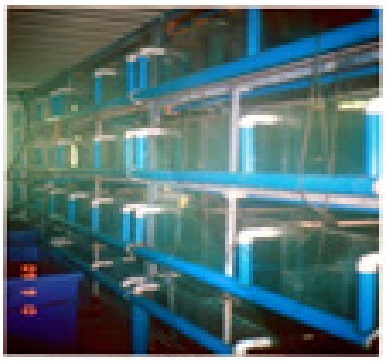
e) Submerged the slides in another round for 1 minute in Xylene-solution

STEP5 **Mounting**

a) Mount the slides with the use of DPX (DPX Mountant for histology is a mixture of distyrene, a plasticizer, and xylene used as a synthetic resin mounting media, that replaces xylene-balsam. It is a popular **all purpose** mounting medium that allows slides to be cleaned easily after setting and will not cause fading when used with a wide variety of stains) medium and cover slides. DPX Mountant is suitable for HE-(Hematoxylin-Eosin)

Cover slips were attached to the slides with DPX mounting solution. DPX mountant for histology is a mixture of distyrene, plasticizer, and xylene

D] Construction of Experimental Facility

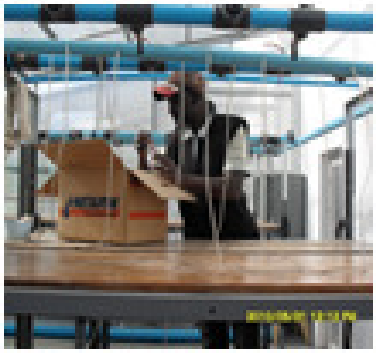


Old glass tanks used to construct experiment unit

Dismantled glass tanks (72), used for the experiment

Steel Platforms that will hold the glass tanks

Steel platforms with wooden planks & inlet pipe lines



Fixing of flexible air and water tubes to inlet pipe lines

Inlet system & air lines

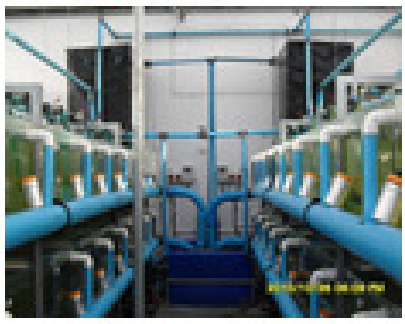
Construction of 3meter Biofilter

Completed Biofilter

Water pumps connected to sumps & biofilter

Mechanical filter connected to sumps & biofilter

Completed system with pumps, sumps, biofilter & glass tanks



Complete functioning system with fish & experimental diets

E] Tables of Statistical Analysis

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa- 1
12:20 Friday, February 15, 2013
The GLM Procedure - Class Level Information

Class	Levels	Values
Tmt	3	C M P
Conc	6	0 0.5 1 5 10 15
Sex	2	F M

Data for Analysis of Length Weight Liver HSI

Number of Observations Read	108
Number of Observations Used	108

Data for Analysis of Depth

Number of Observations Read	108
Number of Observations Used	107

Data for Analysis of Gonad GSI

Number of Observations Read	108
Number of Observations Used	107

NOTE: Variables in each group are consistent with respect to the presence or absence of missing values.

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 2
12:20 Friday, February 15, 2013
The GLM Procedure - Dependent Variable: Length

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	13707.85741	652.75511	12.63	<.0001
Error	86	4444.55000	51.68081		
Corrected Total	107	18152.40741			

R-Square	Coeff Var	Root MSE	Length Mean
0.755154	4.838621	7.188937	148.5741

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	12353.15115	12353.15115	239.03	<.0001
Tmt	2	15.18684	7.59342	0.15	0.8636
Conc	4	380.08673	95.02168	1.84	0.1288
Tmt*Conc	4	295.56001	73.89000	1.43	0.2310
Tmt*Sex	2	44.60408	22.30204	0.43	0.6509
Conc*Sex	4	382.06332	95.51583	1.85	0.1270
Tmt*Conc*Sex	4	237.20528	59.30132	1.15	0.3398

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 3
12:20 Friday, February 15, 2013
The GLM Procedure - Dependent Variable: Weight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	11274.68935	536.88997	7.82	<.0001
Error	86	5901.72820	68.62475		
Corrected Total	107	17176.41754			

R-Square	Coeff Var	Root MSE	Weight Mean
0.656405	15.58337	8.284005	53.15926

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	10318.34352	10318.34352	150.36	<.0001
Tmt	2	8.02188	4.01094	0.06	0.9433
Conc	4	229.14363	57.28591	0.83	0.5068
Tmt*Conc	4	336.20120	84.05030	1.22	0.3063
Tmt*Sex	2	1.47464	0.73732	0.01	0.9893
Conc*Sex	4	261.04988	65.26247	0.95	0.4387
Tmt*Conc*Sex	4	120.45460	30.11365	0.44	0.7802

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 4
12:20 Friday, February 15, 2013

The GLM Procedure - Dependent Variable: Liver

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	3.74642796	0.17840133	2.01	0.0129
Error	86	7.61976000	0.08860186		
Corrected Total	107	11.36618796			

R-Square	Coeff Var	Root MSE	Liver Mean
0.329612	24.18003	0.297661	1.231019

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	1.84877602	1.84877602	20.87	<.0001
Tmt	2	0.50025692	0.25012846	2.82	0.0649
Conc	4	0.34177816	0.08544454	0.96	0.4314
Tmt*Conc	4	0.22875722	0.05718931	0.65	0.6316
Tmt*Sex	2	0.03352920	0.01676460	0.19	0.8280
Conc*Sex	4	0.58410162	0.14602540	1.65	0.1696
Tmt*Conc*Sex	4	0.20922881	0.05230720	0.59	0.6705

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 5
12:20 Friday, February 15, 2013

The GLM Procedure - Dependent Variable: HSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	8.55528237	0.40739440	1.89	0.0220
Error	86	18.58626230	0.21611933		
Corrected Total	107	27.14154467			

R-Square	Coeff Var	Root MSE	HSI Mean
0.315210	19.76184	0.464886	2.352444

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	3.30474557	3.30474557	15.29	0.0002
Tmt	2	1.62926646	0.81463323	3.77	0.0270
Conc	4	0.41457075	0.10364269	0.48	0.7506
Tmt*Conc	4	0.36481111	0.09120278	0.42	0.7924
Tmt*Sex	2	0.28322404	0.14161202	0.66	0.5219
Conc*Sex	4	1.68538019	0.42134505	1.95	0.1095
Tmt*Conc*Sex	4	0.87328425	0.21832106	1.01	0.4068

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 6
12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for Length

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	51.68081
Critical Value of t	1.98793
Least Significant Difference	2.7508
Harmonic Mean of Cell Sizes	53.98148

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	159.073	55	M
B	137.679	53	F

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa -7
12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for Weight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	68.62475
Critical Value of t	1.98793
Least Significant Difference	3.1698

Harmonic Mean of Cell Sizes 53.98148
NOTE: Cell sizes are not equal.
Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	62.754	55	M
B	43.202	53	F

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 8
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for Liver
NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	0.088602
Critical Value of t	1.98793
Least Significant Difference	0.1139
Harmonic Mean of Cell Sizes	53.98148

NOTE: Cell sizes are not equal.
Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	1.35945	55	M
B	1.09774	53	F

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 9
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for HSI
NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	0.216119
Critical Value of t	1.98793
Least Significant Difference	0.1779
Harmonic Mean of Cell Sizes	53.98148

NOTE: Cell sizes are not equal.
Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	2.53064	53	F
B	2.18073	55	M

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 10
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for Length
NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	51.68081
Critical Value of t	1.98793
Least Significant Difference	4.379
Harmonic Mean of Cell Sizes	21.30178

NOTE: Cell sizes are not equal.
Means with the same letter are not significantly different.

t Grouping	Mean	N	Tmt
A	148.771	48	P
A	148.640	50	M
A	147.300	10	C

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 11
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for Weight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	68.62475
Critical Value of t	1.98793
Least Significant Difference	5.046
Harmonic Mean of Cell Sizes	21.30178

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Tmt
A	53.689	48	P
A	52.752	50	M
A	52.656	10	C

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa -12

12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for Liver

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	0.088602
Critical Value of t	1.98793
Least Significant Difference	0.1813
Harmonic Mean of Cell Sizes	21.30178

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Tmt
A	1.37100	10	C
B A	1.27375	48	P
B	1.16200	50	M

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 13

12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for HSI

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	0.216119
Critical Value of t	1.98793
Least Significant Difference	0.2832
Harmonic Mean of Cell Sizes	21.30178

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Tmt
A	2.6580	10	C
B A	2.3989	48	P
B	2.2468	50	M

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 14

12:20 Friday, February 15, 2013

The GLM Procedure - Tests (LSD) for Length

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	51.68081
Critical Value of t	1.98793
Least Significant Difference	4.9179
Harmonic Mean of Cell Sizes	16.88889

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Conc
A	150.900	20	0.5
A	149.850	20	1
A	149.200	20	15
A	147.300	10	0
A	146.895	19	10
A	146.474	19	5

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 15

12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for Weight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	68.62475
Critical Value of t	1.98793
Least Significant Difference	5.667
Harmonic Mean of Cell Sizes	16.88889

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Conc
A	55.035	20	0.5
A	54.588	20	1
A	52.656	10	0
A	52.337	19	5
A	52.224	20	15
A	51.753	19	10

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa -16

12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for Liver

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	0.088602
Critical Value of t	1.98793
Least Significant Difference	0.2036
Harmonic Mean of Cell Sizes	16.88889

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Conc
	A	1.3710	10	0
B	A	1.3205	20	0.5
B	A	1.2200	20	1
B	A	1.1995	19	10
B	A	1.1935	20	15
B		1.1458	19	5

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 17

12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for HSI

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	86
Error Mean Square	0.216119
Critical Value of t	1.98793
Least Significant Difference	0.318
Harmonic Mean of Cell Sizes	16.88889

NOTE: Cell sizes are not equal.
Means with the same letter are not significantly different.

t Grouping	Mean	N	Conc
A	2.6580	10	0
B A	2.4452	20	0.5
B	2.3240	19	10
B	2.2974	20	1
B	2.2914	20	15
B	2.2447	19	5

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 18
12:20 Friday, February 15, 2013
The GLM Procedure - Dependent Variable: Depth

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	1224.344081	58.302099	6.19	<.0001
Error	85	800.216667	9.414314		
Corrected Total	106	2024.560748			

R-Square	Coeff Var	Root MSE	Depth Mean
0.604746	7.960850	3.068275	38.54206

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	1058.283825	1058.283825	112.41	<.0001
Tmt	2	5.443935	2.721968	0.29	0.7496
Conc	4	91.175814	22.793954	2.42	0.0545
Tmt*Conc	4	30.391899	7.597975	0.81	0.5241
Tmt*Sex	2	1.442759	0.721380	0.08	0.9263
Conc*Sex	4	9.846158	2.461539	0.26	0.9019
Tmt*Conc*Sex	4	27.759691	6.939923	0.74	0.5692

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 19
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for Depth
NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	9.414314
Critical Value of t	1.98827
Least Significant Difference	1.18
Harmonic Mean of Cell Sizes	53.45794

NOTE: Cell sizes are not equal.
Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	41.6000	55	M
B	35.3077	52	F

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 20
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for Depth
NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	9.414314
Critical Value of t	1.98827
Least Significant Difference	1.8722
Harmonic Mean of Cell Sizes	21.23494

NOTE: Cell sizes are not equal.
Means with the same letter are not significantly different.

t Grouping	Mean	N	Tmt
A	38.8936	47	P
A	38.5000	10	C

A	38.2200	50	M

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 21
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for Depth

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	9.414314
Critical Value of t	1.98827
Least Significant Difference	2.108
Harmonic Mean of Cell Sizes	16.75102

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Conc
A	39.500	20	0.5
A	39.450	20	1
A	38.944	18	5
A	38.500	10	0
A	37.421	19	10
A	37.400	20	15

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 22
12:20 Friday, February 15, 2013
The GLM Procedure - Dependent Variable: Gonad

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	67.19928512	3.19996596	18.91	<.0001
Error	85	14.38472980	0.16923212		
Corrected Total	106	81.58401491			

R-Square	Coeff Var	Root MSE	Gonad Mean
0.823682	-557.1516	0.411378	-0.073836

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	61.42309752	61.42309752	362.95	<.0001
Tmt	2	0.84820180	0.42410090	2.51	0.0876
Conc	4	0.59345610	0.14836402	0.88	0.4815
Tmt*Conc	4	0.86697747	0.21674437	1.28	0.2840
Tmt*Sex	2	0.01842660	0.00921330	0.05	0.9470
Conc*Sex	4	1.77530804	0.44382701	2.62	0.0403
Tmt*Conc*Sex	4	1.67381758	0.41845440	2.47	0.0505

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 23
12:20 Friday, February 15, 2013
The GLM Procedure - Dependent Variable: GSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	21	100.8539930	4.8025711	33.52	<.0001
Error	85	12.1781042	0.1432718		
Corrected Total	106	113.0320972			

R-Square	Coeff Var	Root MSE	GSI Mean
0.892260	64.44374	0.378513	0.587354

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	95.89686517	95.89686517	669.34	<.0001
Tmt	2	0.91188776	0.45594388	3.18	0.0465
Conc	4	0.60540740	0.15135185	1.06	0.3832
Tmt*Conc	4	0.60177042	0.15044260	1.05	0.3864
Tmt*Sex	2	0.01367062	0.00683531	0.05	0.9534
Conc*Sex	4	1.13382156	0.28345539	1.98	0.1051
Tmt*Conc*Sex	4	1.69057008	0.42264252	2.95	0.0246

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 24
12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for Gonad

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	0.169232
Critical Value of t	1.98827
Least Significant Difference	0.1582
Harmonic Mean of Cell Sizes	53.45794

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	0.70537	52	F
B	-0.81054	55	M

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 25

12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for GSI

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	0.143272
Critical Value of t	1.98827
Least Significant Difference	0.1456
Harmonic Mean of Cell Sizes	53.45794

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	1.56097	52	F
B	-0.33316	55	M

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa -26

12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for Gonad

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	0.169232
Critical Value of t	1.98827
Least Significant Difference	0.251
Harmonic Mean of Cell Sizes	21.23494

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Tmt
	A	0.0350	50	M
B	A	-0.1560	47	P
B		-0.2320	10	C

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 27

12:20 Friday, February 15, 2013

The GLM Procedure - t Tests (LSD) for GSI

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	0.143272
Critical Value of t	1.98827
Least Significant Difference	0.231
Harmonic Mean of Cell Sizes	21.23494

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Tmt
	A	0.7064	50	M
B	A	0.4925	47	P
B		0.4382	10	C

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 28
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for Gonad

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	0.169232
Critical Value of t	1.98827
Least Significant Difference	0.2825
Harmonic Mean of Cell Sizes	16.76471

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Conc
A	0.0376	19	15
A	0.0243	20	0.5
A	-0.0696	19	5
A	-0.1270	20	1
A	-0.1536	19	10
A	-0.2320	10	0

Experiment Ia -60 Day Sampling (Raw Measurements Dec 3, 2010) Pawpaw-Moringa - 29
12:20 Friday, February 15, 2013
The GLM Procedure - t Tests (LSD) for GSI

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	0.143272
Critical Value of t	1.98827
Least Significant Difference	0.2599
Harmonic Mean of Cell Sizes	16.76471

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Conc
	A	0.7074	19	15
B	A	0.6478	20	0.5
B	A	0.6062	19	5
B	A	0.5395	19	10
B	A	0.5151	20	1
B		0.4382	10	0

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction 147. 15:50 Wednesday, August 15, 2012
The GLM Procedure - Class Level Information

Class	Levels	Values
Treat	3	17MT P10M5 P5M10
Sex	2	F M

Number of Observations Read	28
Number of Observations Used	28

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction 148 15:50 Wednesday, August 15, 2012
The GLM Procedure - Dependent Variable: Length

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1799.211905	359.842381	6.22	0.0010
Error	22	1273.466667	57.884848		
Corrected Total	27	3072.678571			

R-Square	Coeff Var	Root MSE	Length Mean
0.585552	4.743484	7.608209	160.3929

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	1497.047802	1497.047802	25.86	<.0001
Treat	2	287.412587	143.706294	2.48	0.1066
Treat*Sex	2	14.751515	7.375758	0.13	0.8810

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction 149 15:50 Wednesday, August 15, 2012

The GLM Procedure - Dependent Variable: Weight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1698.017944	339.603589	5.13	0.0029
Error	22	1457.197867	66.236267		
Corrected Total	27	3155.215811			

R-Square	Coeff Var	Root MSE	Weight Mean
0.538162	12.59903	8.138567	64.59679

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	1114.695425	1114.695425	16.83	0.0005
Treat	2	542.628114	271.314057	4.10	0.0307
Treat*Sex	2	40.694405	20.347203	0.31	0.7386

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interacti 150 15:50 Wednesday, August 15, 2012

The GLM Procedure - Dependent Variable: Depth

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	261.4404762	52.2880952	8.30	0.0002
Error	22	138.6666667	6.3030303		
Corrected Total	27	400.1071429			

R-Square	Coeff Var	Root MSE	Depth Mean
0.653426	6.075743	2.510584	41.32143

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	148.6815018	148.6815018	23.59	<.0001
Treat	2	93.4347319	46.7173660	7.41	0.0035
Treat*Sex	2	19.3242424	9.6621212	1.53	0.2381

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interacti 151 15:50 Wednesday, August 15, 2012

The GLM Procedure - Dependent Variable: Gonad

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	16.49572760	3.29914552	6.97	0.0005
Error	22	10.40648879	0.47302222		
Corrected Total	27	26.90221639			

R-Square	Coeff Var	Root MSE	Gonad Mean
0.613174	-3971.694	0.687766	-0.017317

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	13.85150264	13.85150264	29.28	<.0001
Treat	2	1.52392469	0.76196234	1.61	0.2224
Treat*Sex	2	1.12030028	0.56015014	1.18	0.3248

The GLM Procedure - Dependent Variable: GSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	21.50400478	4.30080096	9.63	<.0001
Error	22	9.82161301	0.44643695		
Corrected Total	27	31.32561778			

R-Square	Coeff Var	Root MSE	GSI Mean
0.686467	154.0844	0.668159	0.433632

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	17.97800621	17.97800621	40.27	<.0001
Treat	2	2.53594917	1.26797459	2.84	0.0799
Treat*Sex	2	0.99004939	0.49502469	1.11	0.3477

The GLM Procedure - Dependent Variable: Liver

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	4.29871143	0.85974229	5.82	0.0014
Error	22	3.25096000	0.14777091		
Corrected Total	27	7.54967143			

R-Square	Coeff Var	Root MSE	Liver Mean
0.569391	24.99646	0.384410	1.537857

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	0.03766220	0.03766220	0.25	0.6187
Treat	2	4.25478378	2.12739189	14.40	0.0001
Treat*Sex	2	0.00626545	0.00313273	0.02	0.9790

The GLM Procedure - Dependent Variable: HSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	8.08931776	1.61786355	7.22	0.0004
Error	22	4.92956520	0.22407115		
Corrected Total	27	13.01888296			

R-Square	Coeff Var	Root MSE	HSI Mean
0.621353	19.77726	0.473362	2.393464

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Sex	1	1.24955949	1.24955949	5.58	0.0275
Treat	2	6.62590286	3.31295143	14.79	<.0001
Treat*Sex	2	0.21385542	0.10692771	0.48	0.6268

The GLM Procedure - t Tests (LSD) for Length

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	57.88485
Critical Value of t	2.07387
Least Significant Difference	5.979

Harmonic Mean of Cell Sizes	13.92857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	167.200	15	M
B	152.538	13	F

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction 156 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Weight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	66.23627
Critical Value of t	2.07387
Least Significant Difference	6.3958
Harmonic Mean of Cell Sizes	13.92857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	70.471	15	M
B	57.819	13	F

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction 157 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Depth

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	6.30303
Critical Value of t	2.07387
Least Significant Difference	1.973
Harmonic Mean of Cell Sizes	13.92857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	43.4667	15	M
B	38.8462	13	F

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa Interaction 158 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Gonad

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	0.473022
Critical Value of t	2.07387
Least Significant Difference	0.5405
Harmonic Mean of Cell Sizes	13.92857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex

A	0.7382	13	F
B	-0.6721	15	M

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa
Interaction 159 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for GSI

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	0.446437
Critical Value of t	2.07387
Least Significant Difference	0.5251
Harmonic Mean of Cell Sizes	13.92857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	1.2944	13	F
B	-0.3123	15	M

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa
Interaction160 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Liver

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	0.147771
Critical Value of t	2.07387
Least Significant Difference	0.3021
Harmonic Mean of Cell Sizes	13.92857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	1.5720	15	M
A	1.4985	13	F

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa
Interaction 161 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for HSI

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	0.224071
Critical Value of t	2.07387
Least Significant Difference	0.372
Harmonic Mean of Cell Sizes	13.92857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Sex
A	2.6204	13	F
B	2.1968	15	M

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa

Interaction 162 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Length

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	57.88485
Critical Value of t	2.07387
Least Significant Difference	7.3445
Harmonic Mean of Cell Sizes	9.230769

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treat
A	164.100	10	17MT
A	158.625	8	P10M5
A	158.100	10	P5M10

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa

Interaction 163 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Weight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	66.23627
Critical Value of t	2.07387
Least Significant Difference	7.8564
Harmonic Mean of Cell Sizes	9.230769

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treat
A	69.921	10	17MT
B	61.898	10	P5M10
B	61.315	8	P10M5

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa

Interaction 164 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Depth

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	6.30303
Critical Value of t	2.07387
Least Significant Difference	2.4236
Harmonic Mean of Cell Sizes	9.230769

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treat
A	43.600	10	17MT
B	40.250	8	P10M5
B	39.900	10	P5M10

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa

Interaction 165 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Gonad

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	0.473022
Critical Value of t	2.07387
Least Significant Difference	0.6639
Harmonic Mean of Cell Sizes	9.230769

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treat
A	0.2286	10	P5M10
A	0.0017	8	P10M5
A	-0.2785	10	17MT

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa
Interaction 166 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for GSI

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	0.446437
Critical Value of t	2.07387
Least Significant Difference	0.645
Harmonic Mean of Cell Sizes	9.230769

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treat
A	0.7282	10	P5M10
A	0.4974	8	P10M5
A	0.0881	10	17MT

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa
Interaction 167 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for Liver

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	0.147771
Critical Value of t	2.07387
Least Significant Difference	0.3711
Harmonic Mean of Cell Sizes	9.230769

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treat
A	2.0500	10	17MT
B	1.3525	8	P10M5
B	1.1740	10	P5M10

Experiment Ib -60 Day Sampling (Raw Measurements Dec 3, 2010)17MT & Pawpaw-Moringa
Interaction 168 15:50 Wednesday, August 15, 2012

The GLM Procedure - t Tests (LSD) for HSI

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	0.224071
Critical Value of t	2.07387
Least Significant Difference	0.457
Harmonic Mean of Cell Sizes	9.230769

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treat
A	3.0377	10	17MT
B	2.1859	8	P10M5
B	1.9153	10	P5M10

The GLM Procedure - Dependent Variable: mLength

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	583.078535	58.307854	2.78	0.0095
Error	44	923.669444	20.992487		
Corrected Total	54	1506.747980			

R-Square	Coeff Var	Root MSE	mLength Mean
0.386978	3.598409	4.581756	127.3273

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	110.8618687	55.4309343	2.64	0.0826
Conc	4	150.4063889	37.6015972	1.79	0.1477
Treat*Conc	4	321.8102778	80.4525694	3.83	0.0093

The GLM Procedure - Dependent Variable: mWeight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	431.171408	43.117141	1.82	0.0851
Error	44	1042.743181	23.698709		
Corrected Total	54	1473.914588			

R-Square	Coeff Var	Root MSE	mWeight Mean
0.292535	11.75608	4.868132	41.40948

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	70.0419346	35.0209673	1.48	0.2393
Conc	4	157.3155389	39.3288847	1.66	0.1765
Treat*Conc	4	203.8139341	50.9534835	2.15	0.0905

The GLM Procedure - Dependent Variable: mDepth

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	75.6093434	7.5609343	1.42	0.2036
Error	44	234.3583333	5.3263258		
Corrected Total	54	309.9676768			

R-Square	Coeff Var	Root MSE	mDepth Mean
0.243927	6.109675	2.307883	37.77424

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	19.88767677	9.94383838	1.87	0.1666
Conc	4	6.89527778	1.72381944	0.32	0.8606
Treat*Conc	4	48.82638889	12.20659722	2.29	0.0746

Experiment II 60 Day Sampling (Raw Measurements Jun 20, 2011) males 192
15:37 Thursday, August 16, 2012

The GLM Procedure - Dependent Variable: mGonad

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	3.92755600	0.39275560	1.83	0.0823
Error	44	9.42146536	0.21412421		
Corrected Total	54	13.34902137			

R-Square	Coeff Var	Root MSE	mGonad Mean
0.294221	-18.28968	0.462736	-2.530036

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	1.03898147	0.51949073	2.43	0.1001
Conc	4	0.57820776	0.14455194	0.68	0.6128
Treat*Conc	4	2.31036677	0.57759169	2.70	0.0428

The GLM Procedure - Dependent Variable: mGSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	3.82133766	0.38213377	1.66	0.1203
Error	44	10.10497887	0.22965861		
Corrected Total	54	13.92631653			

R-Square	Coeff Var	Root MSE	mGSI Mean
0.274397	-29.37253	0.479227	-1.631549

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	0.69390818	0.34695409	1.51	0.2320
Conc	4	0.52544967	0.13136242	0.57	0.6843
Treat*Conc	4	2.60197981	0.65049495	2.83	0.0356

The GLM Procedure - Dependent Variable: mLiver

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	0.94661035	0.09466104	1.67	0.1192
Error	44	2.49682000	0.05674591		
Corrected Total	54	3.44343035			

R-Square	Coeff Var	Root MSE	mLiver Mean
0.274903	18.77515	0.238214	1.268773

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	0.24937091	0.12468545	2.20	0.1232
Conc	4	0.49239328	0.12309832	2.17	0.0882
Treat*Conc	4	0.20484617	0.05121154	0.90	0.4708

Experiment II 60 Day Sampling (Raw Measurements Jun 20, 2011) males 195
15:37 Thursday, August 16, 2012

The GLM Procedure - Dependent Variable: mHSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	6.76530744	0.67653074	3.32	0.0027
Error	44	8.95756632	0.20358105		
Corrected Total	54	15.72287376			

R-Square	Coeff Var	Root MSE	mHSI Mean
0.430284	14.69437	0.451200	3.070561

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	2.18757189	1.09378595	5.37	0.0082
Conc	4	2.24120854	0.56030214	2.75	0.0397
Treat*Conc	4	2.33652700	0.58413175	2.87	0.0339

The GLM Procedure - Least Squares Means

mLength LSMEAN

Treat	Conc	LSMEAN	Number
C	0	123.716667	1
M	0.5	124.316667	2
M	1	126.633333	3
M	2	126.116667	4
M	5	129.833333	5
M	10	135.966667	6
P	0.5	127.633333	7
P	1	127.633333	8
P	2	125.750000	9
P	5	128.450000	10
P	10	124.550000	11

Least Squares Means for effect Treat*Conc

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: mLength

i/j	1	2	3	4	5	6
1		0.8369	0.3197	0.4120	0.0405	0.0001
2	0.8369		0.4283	0.5377	0.0635	0.0002
3	0.3197	0.4283		0.8593	0.2755	0.0024
4	0.4120	0.5377	0.8593		0.2063	0.0014
5	0.0405	0.0635	0.2755	0.2063		0.0400
6	0.0001	0.0002	0.0024	0.0014	0.0400	
7	0.1834	0.2586	0.7317	0.6033	0.4518	0.0062
8	0.1834	0.2586	0.7317	0.6033	0.4518	0.0062
9	0.4866	0.6233	0.7619	0.8999	0.1658	0.0010
10	0.1095	0.1608	0.5340	0.4250	0.6355	0.0128
11	0.7750	0.9362	0.4760	0.5915	0.0751	0.0003

Dependent Variable: mLength

i/j	7	8	9	10	11
1	0.1834	0.1834	0.4866	0.1095	0.7750
2	0.2586	0.2586	0.6233	0.1608	0.9362
3	0.7317	0.7317	0.7619	0.5340	0.4760
4	0.6033	0.6033	0.8999	0.4250	0.5915
5	0.4518	0.4518	0.1658	0.6355	0.0751
6	0.0062	0.0062	0.0010	0.0128	0.0003
7		1.0000	0.5191	0.7794	0.2931
8	1.0000		0.5191	0.7794	0.2931
9	0.5191	0.5191		0.3565	0.6808
10	0.7794	0.7794	0.3565		0.1852
11	0.2931	0.2931	0.6808	0.1852	

mWeight LSMEAN

Treat	Conc	LSMEAN	Number
C	0	38.9841667	1
M	0.5	39.2835000	2
M	1	41.6083333	3
M	2	40.5620000	4
M	5	42.7173333	5
M	10	48.4300000	6
P	0.5	40.0855000	7

P	1	40.4673333	8
P	2	39.4943333	9
P	5	44.8696667	10
P	10	39.0021667	11

Least Squares Means for effect Treat*Conc
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: mWeight

i/j	1	2	3	4	5	6
1		0.9230	0.3987	0.6109	0.2318	0.0037
2	0.9230		0.4542	0.6800	0.2708	0.0048
3	0.3987	0.4542		0.7356	0.7204	0.0319
4	0.6109	0.6800	0.7356		0.4876	0.0141
5	0.2318	0.2708	0.7204	0.4876		0.0702
6	0.0037	0.0048	0.0319	0.0141	0.0702	
7	0.7223	0.7957	0.6233	0.8777	0.3973	0.0095
8	0.6324	0.7025	0.7127	0.9756	0.4688	0.0131
9	0.8692	0.9457	0.4959	0.7304	0.3009	0.0058
10	0.0625	0.0764	0.2953	0.1688	0.4882	0.2538
11	0.9954	0.9276	0.4019	0.6149	0.2340	0.0037

Dependent Variable: mWeight

i/j	7	8	9	10	11
1	0.7223	0.6324	0.8692	0.0625	0.9954
2	0.7957	0.7025	0.9457	0.0764	0.9276
3	0.6233	0.7127	0.4959	0.2953	0.4019
4	0.8777	0.9756	0.7304	0.1688	0.6149
5	0.3973	0.4688	0.3009	0.4882	0.2340
6	0.0095	0.0131	0.0058	0.2538	0.0037
7		0.9019	0.8486	0.1274	0.7266
8	0.9019		0.7535	0.1598	0.6365
9	0.8486	0.7535		0.0878	0.8737
10	0.1274	0.1598	0.0878		0.0632
11	0.7266	0.6365	0.8737	0.0632	

mDepth LSMEAN

Treat	Conc	LSMEAN	Number
C	0	5.8833333	1
M	0.5	37.7500000	2
M	1	38.0666667	3
M	2	39.1666667	4
M	5	35.8666667	5
M	10	38.6333333	6
P	0.5	37.6500000	7
P	1	36.9166667	8
P	2	37.8833333	9
P	5	39.8666667	10
P	10	37.8333333	11

Least Squares Means for effect Treat*Conc
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: mDepth

i/j	1	2	3	4	5	6
1		0.2077	0.1418	0.0295	0.9909	0.0662

2	0.2077		0.8293	0.3371	0.2037	0.5482
3	0.1418	0.8293		0.4551	0.1389	0.6997
4	0.0295	0.3371	0.4551		0.0288	0.7166
5	0.9909	0.2037	0.1389	0.0288		0.0646
6	0.0662	0.5482	0.6997	0.7166	0.0646	
7	0.2326	0.9457	0.7766	0.3044	0.2283	0.5040
8	0.4827	0.5710	0.4350	0.1304	0.4757	0.2459
9	0.1776	0.9276	0.9006	0.3841	0.1741	0.6099
10	0.0091	0.1541	0.2241	0.6339	0.0088	0.4027
11	0.1884	0.9547	0.8737	0.3660	0.1848	0.5864

Dependent Variable: mDepth

i/j	7	8	9	10	11
1	0.2326	0.4827	0.1776	0.0091	0.1884
2	0.9457	0.5710	0.9276	0.1541	0.9547
3	0.7766	0.4350	0.9006	0.2241	0.8737
4	0.3044	0.1304	0.3841	0.6339	0.3660
5	0.2283	0.4757	0.1741	0.0088	0.1848
6	0.5040	0.2459	0.6099	0.4027	0.5864
7		0.6179	0.8737	0.1360	0.9006
8	0.6179		0.5113	0.0494	0.5332
9	0.8737	0.5113		0.1811	0.9728
10	0.1360	0.0494	0.1811		0.1706
11	0.9006	0.5332	0.9728	0.1706	

mGonad LSMEAN

Treat	Conc	LSMEAN	Number
C	0	-2.90440142	1
M	0.5	-2.34066359	2
M	1	-2.91855222	3
M	2	-2.17154859	4
M	5	-2.10114156	5
M	10	-2.56492372	6
P	0.5	-2.65046697	7
P	1	-2.27811686	8
P	2	-2.76953152	9
P	5	-2.50624058	10
P	10	-2.62481443	11

Least Squares Means for effect Treat*Conc

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: mGonad

i/j	1	2	3	4	5	6
1		0.0605	0.9617	0.0161	0.0087	0.2523
2	0.0605		0.0546	0.5663	0.4175	0.4476
3	0.9617	0.0546		0.0142	0.0077	0.2334
4	0.0161	0.5663	0.0142		0.8110	0.1858
5	0.0087	0.4175	0.0077	0.8110		0.1202
6	0.2523	0.4476	0.2334	0.1858	0.1202	
7	0.3903	0.2956	0.3646	0.1089	0.0672	0.7714
8	0.0379	0.8318	0.0340	0.7175	0.5485	0.3324
9	0.6472	0.1499	0.6132	0.0470	0.0273	0.4881
10	0.1806	0.5744	0.1659	0.2590	0.1733	0.8420

11	0.3446	0.3369	0.3210	0.1286	0.0804	0.8388

Dependent Variable: mGonad

i/j	7	8	9	10	11
1	0.3903	0.0379	0.6472	0.1806	0.3446
2	0.2956	0.8318	0.1499	0.5744	0.3369
3	0.3646	0.0340	0.6132	0.1659	0.3210
4	0.1089	0.7175	0.0470	0.2590	0.1286
5	0.0672	0.5485	0.0273	0.1733	0.0804
6	0.7714	0.3324	0.4881	0.8420	0.8388
7		0.2100	0.6861	0.6246	0.9306
8	0.2100		0.1002	0.4399	0.2425
9	0.6861	0.1002		0.3732	0.6234
10	0.6246	0.4399	0.3732		0.6873
11	0.9306	0.2425	0.6234	0.6873	

The GLM Procedure - Least Squares Means

LSMEAN

Treat	Conc	mGSI LSMEAN	Number
C	0	-1.94967616	1
M	0.5	-1.39594806	2
M	1	-2.01879010	3
M	2	-1.25061283	4
M	5	-1.24498002	5
M	10	-1.82635587	6
P	0.5	-1.72486140	7
P	1	-1.36164981	8
P	2	-1.83212612	9
P	5	-1.68106979	10
P	10	-1.66096399	11

Least Squares Means for effect Treat*Conc

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: mGSI

i/j	1	2	3	4	5	6
1		0.0745	0.8207	0.0259	0.0247	0.6861
2	0.0745		0.0458	0.6339	0.6209	0.1626
3	0.8207	0.0458		0.0149	0.0142	0.5288
4	0.0259	0.6339	0.0149		0.9853	0.0641
5	0.0247	0.6209	0.0142	0.9853		0.0616
6	0.6861	0.1626	0.5288	0.0641	0.0616	
7	0.4622	0.2837	0.3375	0.1248	0.1205	0.7393
8	0.0588	0.9104	0.0356	0.7159	0.7021	0.1324
9	0.7000	0.1572	0.5412	0.0615	0.0592	0.9849
10	0.3803	0.3520	0.2712	0.1626	0.1573	0.6341
11	0.3460	0.3867	0.2441	0.1827	0.1769	0.5880

Dependent Variable: mGSI

i/j	7	8	9	10	11
1	0.4622	0.0588	0.7000	0.3803	0.3460
2	0.2837	0.9104	0.1572	0.3520	0.3867
3	0.3375	0.0356	0.5412	0.2712	0.2441
4	0.1248	0.7159	0.0615	0.1626	0.1827
5	0.1205	0.7021	0.0592	0.1573	0.1769
6	0.7393	0.1324	0.9849	0.6341	0.5880

7		0.2372	0.7251	0.8858	0.8340
8	0.2372		0.1278	0.2977	0.3288
9	0.7251	0.1278		0.6207	0.5751
10	0.8858	0.2977	0.6207		0.9474
11	0.8340	0.3288	0.5751	0.9474	

mLiver LSMEAN

Treat	Conc	LSMEAN	Number
C	0	1.45950000	1
M	0.5	1.25050000	2
M	1	1.26166667	3
M	2	1.38016667	4
M	5	1.44033333	5
M	10	1.07283333	6
P	0.5	1.23183333	7
P	1	1.11616667	8
P	2	1.12700000	9
P	5	1.41883333	10
P	10	1.19766667	11

Least Squares Means for effect Treat*Conc
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: mLiver

i/j	1	2	3	4	5	6
1		0.1724	0.1960	0.6011	0.8993	0.0138
2	0.1724		0.9413	0.3941	0.2143	0.2446
3	0.1960	0.9413		0.4358	0.2420	0.2167
4	0.6011	0.3941	0.4358		0.6916	0.0474
5	0.8993	0.2143	0.2420	0.6916		0.0188
6	0.0138	0.2446	0.2167	0.0474	0.0188	
7	0.1379	0.9020	0.8439	0.3302	0.1734	0.2970
8	0.0276	0.3774	0.3394	0.0867	0.0370	0.7750
9	0.0326	0.4168	0.3763	0.1000	0.0434	0.7209
10	0.7885	0.2699	0.3026	0.7986	0.8872	0.0265
11	0.0892	0.7275	0.6731	0.2322	0.1144	0.4118

Dependent Variable: mLiver

i/j	7	8	9	10	11
1	0.1379	0.0276	0.0326	0.7885	0.0892
2	0.9020	0.3774	0.4168	0.2699	0.7275
3	0.8439	0.3394	0.3763	0.3026	0.6731
4	0.3302	0.0867	0.1000	0.7986	0.2322
5	0.1734	0.0370	0.0434	0.8872	0.1144
6	0.2970	0.7750	0.7209	0.0265	0.4118
7		0.4467	0.4902	0.2211	0.8216
8	0.4467		0.9430	0.0507	0.5913
9	0.4902	0.9430		0.0592	0.6414
10	0.2211	0.0507	0.0592		0.1492
11	0.8216	0.5913	0.6414	0.1492	

LSMEAN

Treat	Conc	mHSI LSMEAN	Number
C	0	3.69981667	1
M	0.5	3.16635000	2
M	1	3.00810000	3

M	2	3.33438333	4
M	5	3.36780000	5
M	10	2.23145000	6
P	0.5	3.02970000	7
P	1	2.88181667	8
P	2	2.87640000	9
P	5	3.14585000	10
P	10	3.03450000	11

The GLM Procedure - Least Squares Means

Least Squares Means for effect Treat*Conc

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: mHSI

i/j	1	2	3	4	5	6
1		0.0682	0.0195	0.2070	0.2509	<.0001
2	0.0682		0.5820	0.5590	0.4839	0.0021
3	0.0195	0.5820		0.2591	0.2141	0.0093
4	0.2070	0.5590	0.2591		0.9073	0.0004
5	0.2509	0.4839	0.2141	0.9073		0.0003
6	<.0001	0.0021	0.0093	0.0004	0.0003	
7	0.0234	0.6344	0.9400	0.2915	0.2425	0.0076
8	0.0063	0.3242	0.6603	0.1199	0.0956	0.0276
9	0.0060	0.3151	0.6467	0.1157	0.0921	0.0288
10	0.0586	0.9431	0.6317	0.5123	0.4409	0.0025
11	0.0244	0.6463	0.9267	0.2991	0.2491	0.0073

Dependent Variable: mHSI

i/j	7	8	9	10	11
1	0.0234	0.0063	0.0060	0.0586	0.0244
2	0.6344	0.3242	0.3151	0.9431	0.6463
3	0.9400	0.6603	0.6467	0.6317	0.9267
4	0.2915	0.1199	0.1157	0.5123	0.2991
5	0.2425	0.0956	0.0921	0.4409	0.2491
6	0.0076	0.0276	0.0288	0.0025	0.0073
7		0.6069	0.5938	0.6860	0.9867
8	0.6069		0.9849	0.3599	0.5953
9	0.5938	0.9849		0.3502	0.5824
10	0.6860	0.3599	0.3502		0.6983
11	0.9867	0.5953	0.5824	0.6983	

Experiment II -60 Day Sampling (Raw Measurements Jun 20, 2011) Females 60

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The GLM Procedure - Class Level Information

Class	Levels	Values
Treat	3	C M P
Conc	6	0 0.5 1 2 5 10

Number of Observations Read 42

Number of Observations Used 42

The GLM Procedure - Dependent Variable: mLength

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	315.207870	31.520787	0.70	0.7133
Error	31	1387.384722	44.754346		
Corrected Total	41	1702.592593			

R-Square	Coeff Var	Root MSE	mLength Mean

0.185134	5.488498	6.689869	121.8889	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	80.6695326	40.3347663	0.90	0.4164
Conc	4	47.0467693	11.7616923	0.26	0.8995
Treat*Conc	4	187.4915684	46.8728921	1.05	0.3989

The GLM Procedure - Dependent Variable: mWeight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	256.459746	25.645975	0.64	0.7673
Error	31	1239.711405	39.990690		
Corrected Total	41	1496.171151			

R-Square	Coeff Var	Root MSE	mWeight Mean
0.171411	17.43069	6.323819	36.27980

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	161.3567352	80.6783676	2.02	0.1501
Conc	4	24.6119831	6.1529958	0.15	0.9598
Treat*Conc	4	70.4910275	17.6227569	0.44	0.7782

The GLM Procedure - Dependent Variable: mDepth

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	118.9029101	11.8902910	0.98	0.4809
Error	31	376.4972222	12.1450717		
Corrected Total	41	495.4001323			

R-Square	Coeff Var	Root MSE	mDepth Mean
0.240014	9.935677	3.484978	35.07540

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	54.59678131	27.29839065	2.25	0.1226
Conc	4	34.09047754	8.52261939	0.70	0.5967
Treat*Conc	4	30.21565121	7.55391280	0.62	0.6503

The GLM Procedure - Dependent Variable: mGonad

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	4.47329324	0.44732932	0.25	0.9874
Error	31	55.30612950	1.78406869		
Corrected Total	41	59.77942274			

R-Square	Coeff Var	Root MSE	mGonad Mean
0.074830	-247.8558	1.335690	-0.538898

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	0.05987355	0.02993678	0.02	0.9834
Conc	4	3.65911412	0.91477853	0.51	0.7268
Treat*Conc	4	0.75430557	0.18857639	0.11	0.9797

The GLM Procedure - Dependent Variable: mGSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	3.84426170	0.38442617	0.21	0.9931
Error	31	55.61031278	1.79388106		
Corrected Total	41	59.45457448			

R-Square	Coeff Var	Root MSE	mGSI Mean
0.064659	283.5544	1.339358	0.472346

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	0.14989916	0.07494958	0.04	0.9591
Conc	4	3.23895254	0.80973814	0.45	0.7706
Treat*Conc	4	0.45540999	0.11385250	0.06	0.9922

Experiment II -60 Day Sampling (Raw Measurements Jun 20, 2011) Female 66

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The GLM Procedure - Dependent Variable: mLiver

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	0.49552188	0.04955219	0.43	0.9186
Error	31	3.54465722	0.11434378		
Corrected Total	41	4.04017910			

R-Square	Coeff Var	Root MSE	mLiver Mean
0.122648	33.84430	0.338148	0.999127

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	0.00478309	0.00239155	0.02	0.9793
Conc	4	0.20234457	0.05058614	0.44	0.7770
Treat*Conc	4	0.28839422	0.07209856	0.63	0.6444

The GLM Procedure - Dependent Variable: mHSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	4.45035498	0.44503550	1.00	0.4650
Error	31	13.80049674	0.44517731		
Corrected Total	41	18.25085172			

R-Square	Coeff Var	Root MSE	mHSI Mean
0.243844	24.42070	0.667216	2.732175

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Treat	2	1.28911617	0.64455809	1.45	0.2505
Conc	4	1.40929015	0.35232254	0.79	0.5397
Treat*Conc	4	1.75194866	0.43798717	0.98	0.4307

New Experiment II -60 Day Sampling of Growth Study Ia

Obs	Conc	Type	TYPE	FREQ	mLength	mWeight	mDepth	llength	lweight	ldepth
1	0.0	C	3	18	217.611	199.797	68.3889	122.75	38.2450	36.40
2	0.5	M	3	16	209.688	179.666	64.5625	120.35	35.0275	36.45
3	0.5	P	3	19	213.105	180.045	65.0526	125.60	37.8325	37.45
4	1.0	M	3	20	207.150	166.228	63.7000	118.50	35.1850	36.15
5	1.0	P	3	18	213.278	178.839	65.3333	124.80	38.5640	38.45
6	2.0	M	3	18	206.444	165.389	63.1111	120.45	35.4185	36.00
7	2.0	P	3	19	207.000	159.179	61.8421	124.35	40.7700	38.80
8	5.0	M	3	17	206.647	177.506	66.1765	127.75	42.8235	37.55
9	5.0	P	3	16	214.563	184.547	65.3125	121.70	38.1470	5.80
10	10.0	M	3	18	206.278	166.711	63.1111	126.00	39.9190	36.25
11	10.0	P	3	17	213.529	188.168	67.6471	120.35	35.5730	35.90

The GLM Procedure - Class Level Information

Class	Levels	Values
Conc	6	0 0.5 1 2 5 10
Type	3	C M P

Number of Observations Read 11

Number of Observations Used 11

The GLM Procedure - Dependent Variable: mLength

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	145.4593346	24.2432224	5.23	0.0656
Error	4	18.5418561	4.6354640		
Corrected Total	10	164.0011907			

R-Square	Coeff Var	Root MSE	mLength Mean
0.886941	1.022900	2.153013	210.4812

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Conc	5	81.61128476	16.32225695	3.52	0.1232
Type	1	63.84804988	63.84804988	13.77	0.0206

The GLM Procedure - Dependent Variable: mWeight

Source	DF	Sum Squares	Mean Square	F Value	Pr > F
Model	6	1166.238157	194.373026	3.39	0.1288
Error	4	229.410032	57.352508		
Corrected Total	10	1395.648189			

R-Square	Coeff Var	Root MSE	mWeight Mean
0.835625	4.280650	7.573144	176.9158

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Conc	5	1041.784039	208.356808	3.63	0.1177
Type	1	124.454117	124.454117	2.17	0.2147

The GLM Procedure - Dependent Variable: mDepth

Source	DF	Sum Squares	Mean Square	F Value	Pr > F
Model	6	28.15282854	4.69213809	1.73	0.3107
Error	4	10.87096225	2.71774056		
Corrected Total	10	39.02379079			

R-Square	Coeff Var	Root MSE	mDepth Mean
0.721427	2.538949	1.648557	64.93070

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Conc	5	26.10396608	5.22079322	1.92	0.2734
Type	1	2.04886246	2.04886246	0.75	0.4342

The GLM Procedure - t Tests (LSD) for mLength

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	4.635464
Critical Value of t	2.77645
Least Significant Difference	6.4567
Harmonic Mean of Cell Sizes	1.714286

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Conc
A	217.611	1	0
B A	211.396	2	0.5
B	210.605	2	5
B	210.214	2	1
B	209.904	2	10
B	206.722	2	2

The GLM Procedure - t Tests (LSD) for mWeight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	57.35251
Critical Value of t	2.77645

Least Significant Difference	22.711
Harmonic Mean of Cell Sizes	1.714286

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Conc
	A	199.797	1	0
B	A	181.027	2	5
B	A	179.855	2	0.5
B	A	177.439	2	10
B		172.533	2	1
B		162.284	2	2

The GLM Procedure - t Tests (LSD) for mDepth

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	2.717741
Critical Value of t	2.77645
Least Significant Difference	4.9439
Harmonic Mean of Cell Sizes	1.714286

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Conc
	A	68.389	1	0
B	A	65.744	2	5
B	A	65.379	2	10
B	A	64.808	2	0.5
B	A	64.517	2	1
B		62.477	2	2

The GLM Procedure - t Tests (LSD) for mLength

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	4.635464
Critical Value of t	2.77645
Least Significant Difference	5.775
Harmonic Mean of Cell Sizes	2.142857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Type
	A	217.611	1	C
B	A	212.295	5	P
B		207.241	5	M

The GLM Procedure - t Tests (LSD) for mWeight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	57.35251
Critical Value of t	2.77645
Least Significant Difference	20.313

Harmonic Mean of Cell Sizes	2.142857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Type
A	199.797	1	C
B	178.155	5	P
B	171.100	5	M

The GLM Procedure - t Tests (LSD) for mDepth

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	2.717741
Critical Value of t	2.77645
Least Significant Difference	4.4219
Harmonic Mean of Cell Sizes	2.142857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Type
A	68.389	1	C
A	65.038	5	P
A	64.132	5	M

The GLM Procedure - Class Level Information

Class	Levels	Values
Conc	5	0.5 1 2 5 10
Type	2	M P

Number of Observations Read	10
Number of Observations Used	10

The GLM Procedure - Dependent Variable: mLength

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	97.6734345	16.2789058	4.69	0.1162
Error	3	10.4080541	3.4693514		
Corrected Total	9	108.0814886			

R-Square	Coeff Var	Root MSE	mLength Mean
0.903702	0.887942	1.862619	209.7682

Source	DF	Type I SS	Mean Square	F Value	Pr > F
mIlength	1	1.11128202	1.11128202	0.32	0.6110
Conc	4	27.58960524	6.89740131	1.99	0.2995
Type	1	68.97254729	68.97254729	19.88	0.0210

The GLM Procedure - Least Squares Means

Conc	mLength LSMEAN	Number
0.5	211.393068	1
1	209.771550	2
2	206.528388	3
5	211.181312	4
10	209.966549	5

Least Squares Means for effect Conc
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: mLength

i/j	1	2	3	4	5
1		0.4528	0.0800	0.9183	0.4996
2	0.4528		0.1812	0.5274	0.9244
3	0.0800	0.1812		0.0949	0.1633
4	0.9183	0.5274	0.0949		0.5666
5	0.4996	0.9244	0.1633	0.5666	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

H0:LSMean1=
mLength LSMean2

Type	LSMEAN	Pr > |t|
M	207.117104	0.0210
P	212.419243	

The GLM Procedure - Class Level Information

Class	Levels	Values
Conc	5	0.5 1 2 5 10
Type	2	M P

Number of Observations Read 10
Number of Observations Used 10

The GLM Procedure - Dependent Variable: mWeight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	692.2004130	115.3667355	2.71	0.2211
Error	3	127.5303192	42.5101064		
Corrected Total	9	819.7307322			

R-Square	Coeff Var	Root MSE	mWeight Mean
0.844424	3.733646	6.519977	174.6276

Source	DF	Type I SS	Mean Square	F Value	Pr > F
mIweight	1	26.8247180	26.8247180	0.63	0.4850
Conc	4	514.5588816	128.6397204	3.03	0.1949
Type	1	150.8168134	150.8168134	3.55	0.1561

The GLM Procedure - Least Squares Means
mWeight LSMEAN

Conc	LSMEAN	Number
0.5	177.571220	1
1	170.927857	2
2	162.540787	3
5	184.933910	4
10	177.164293	5

Least Squares Means for effect Conc
Pr > |t| for H0: LSMean(i)=LSMean(j) -Dependent Variable: mWeight

i/j	1	2	3	4	5
1		0.3842	0.1114	0.4068	0.9550
2	0.3842		0.2952	0.1559	0.4129
3	0.1114	0.2952		0.0482	0.1110
4	0.4068	0.1559	0.0482		0.3513

5	0.9550	0.4129	0.1110	0.3513

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

H0:LSMean1= mWeight LSMean2

Type	LSMEAN	Pr > |t|
M	170.716145	0.1561
P	178.539082	

The GLM Procedure - Class Level Information

Class	Levels	Values
Conc	5	0.5 1 2 5 10
Type	2	M P

Number of Observations Read	10
Number of Observations Used	10

The GLM Procedure - Dependent Variable: mDepth

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	15.31734883	2.55289147	0.73	0.6631
Error	3	10.55147193	3.51715731		
Corrected Total	9	25.86882076			

R-Square	Coeff Var	Root MSE	mDepth Mean
0.592116	2.903789	1.875409	64.58488

Source	DF	Type I SS	Mean Square	F Value	Pr > F
mIdepth	1	1.22693244	1.22693244	0.35	0.5963
Conc	4	11.73309048	2.93327262	0.83	0.5831
Type	1	2.35732590	2.35732590	0.67	0.4729

The GLM Procedure - Least Squares Means

mDepth LSMEAN

Conc	LSMEAN	Number
0.5	64.8224594	1
1	64.6060286	2
2	62.5872468	3
5	65.7008681	4
10	65.2078079	5

Least Squares Means for effect Conc

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: mDepth

i/j	1	2	3	4	5
1		0.9161	0.3247	0.6730	0.8577
2	0.9161		0.3609	0.6096	0.7897
3	0.3247	0.3609		0.2075	0.2998
4	0.6730	0.6096	0.2075		0.8142
5	0.8577	0.7897	0.2998	0.8142	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

H0:LSMean1= mDepth LSMean2

Type	LSMEAN	Pr > |t|
M	64.0471319	0.4729
P	65.1226324	

Growth Experiment II - Female

The GLM Procedure - Class Level Information

Class	Levels	Values
Conc	6	0 0.5 1 2 5 10
Type	3	C M P

Number of Observations Read 196
Number of Observations Used 196

The GLM Procedure - Dependent Variable: Length

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	2933.74895	293.37489	0.68	0.7420
Error	185	79793.77657	431.31771		
Corrected Total	195	82727.52551			

R-Square	Coeff Var	Root MSE	Length Mean
0.035463	9.870190	20.76819	210.4133

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Conc	5	1497.527560	299.505512	0.69	0.6283
Type	1	1105.465019	1105.465019	2.56	0.1111
Conc*Type	4	330.756366	82.689092	0.19	0.9425

The GLM Procedure - Dependent Variable: Weight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	25391.9827	2539.1983	1.19	0.2973
Error	185	393363.6455	2126.2900		
Corrected Total	195	418755.6282			

R-Square	Coeff Var	Root MSE	Weight Mean
0.060637	26.11588	46.11171	176.5658

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Conc	5	19093.69608	3818.73922	1.80	0.1156
Type	1	2178.92995	2178.92995	1.02	0.3127
Conc*Type	4	4119.35667	1029.83917	0.48	0.7472

The GLM Procedure - Dependent Variable: Depth

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	705.06606	70.50661	1.19	0.2973
Error	185	10923.23496	59.04451		
Corrected Total	95	11628.30102			

R-Square	Coeff Var	Root MSE	Depth Mean
0.060634	11.84298	7.684043	64.88265

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Conc	5	476.7854775	95.3570955	1.62	0.1580
Type	1	37.0202192	37.0202192	0.63	0.4295
Conc*Type	4	191.2603650	47.8150913	0.81	0.5203

The GLM Procedure - t Tests (LSD) for Length

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	185
Error Mean Square	431.3177
Critical Value of t	1.97287
Least Significant Difference	10.482
Harmonic Mean of Cell Sizes	30.55857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Conc
	A	217.611	18	0
B	A	211.543	35	0.5
B	A	210.485	33	5
B	A	210.053	38	1
B	A	209.800	35	10
B		206.730	37	2

The GLM Procedure - t Tests (LSD) for Weight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	185
Error Mean Square	2126.29
Critical Value of t	1.97287
Least Significant Difference	23.273
Harmonic Mean of Cell Sizes	30.55857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Conc
	A	199.80	18	0
B	A	180.92	33	5
B	A	179.87	35	0.5
B	A	177.13	35	10
B		172.20	38	1
B		162.20	37	2

The GLM Procedure - t Tests (LSD) for Depth

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	185
Error Mean Square	59.04451
Critical Value of t	1.97287
Least Significant Difference	3.8783
Harmonic Mean of Cell Sizes	30.55857

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping		Mean	N	Conc
	A	68.389	18	0
B	A	65.758	33	5
B	A	65.314	35	10
B	A	64.829	35	0.5
B		64.474	38	1
B		62.459	37	2

The GLM Procedure - t Tests (LSD) for Length

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	185
Error Mean Square	431.3177
Critical Value of t	1.97287

Least Significant Difference	9.3449
Harmonic Mean of Cell Sizes	38.448

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Type
A	217.611	18	C
B A	212.180	89	P
B	207.191	89	M

The GLM Procedure - t Tests (LSD) for Weight

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	185
Error Mean Square	2126.29
Critical Value of t	1.97287
Least Significant Difference	20.749
Harmonic Mean of Cell Sizes	38.448

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Type
A	199.80	18	C
B	177.71	89	P
B	170.73	89	M

The GLM Procedure - t Tests (LSD) for Depth

NOTE: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	185
Error Mean Square	59.04451
Critical Value of t	1.97287
Least Significant Difference	3.4575
Harmonic Mean of Cell Sizes	38.448

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Type
A	68.389	18	C
B A	64.966	89	P
B	64.090	89	M

F] Limitations and Constraints to the Experiment

1 LIMITATIONS TO THE WORK

-Unavailability of fish at the start of the experiment

-Size Range

-Age and conditions of rearing fish

-Operational difficulties

-Preparation of feed fortnightly

2 CONSTRAINTS TO THE EXPERIMENT

Fish Selection

The experiment faced a number of challenges which nearly marred its smooth take off, which include:

-By the time the experiment commenced available fish produced in December 2009 which would have provided uniform size for the study had been sold out to farmers by the Technical Manager citing lack of space in the existing facilities, and also overcrowding stretching the carrying capacity of the available space in the systems (grey system and raceway)

-The rest were sent to the tunnel (pond system made from tarpaulin, another holding facility for rejected fish and those not being used for experiments) to mix with stunting and diseased fish, suffering massive mortality

-Similarly fish produced for experiment II suffered the same fate of limited holding facility and desire to disposed them off by the Technical Manager

-This actually placed a strain on selection of fish for the experiment.

Experiment I

-It took more than 8months (September 2009 - July 2010) to complete experimental unit

-When it became fully operational in August 2010 there were no or very small fish for the experiment, particularly those specifically breed for the experiment either disposed off or dead

- Fish for stocking were not enough, so had to rely on those from the tunnel for stoking, hence the wide size range, 20-40g

-It took 20days to obtain sexually mature fish even within that wide size range to stock 70 tanks

-Because fish kept in the tunnel were overcrowded, they appeared stunted. So when placed in more spacious glass tanks, the fish quickly showed reproductive behavior (i.e. dark bodied fish developed within 3-days of stocking trying to establish territory)

-Most fish had poorly developed urogenital (or urinogenital) papilla and fins (dorsal and anal) which were the main external morphological characteristics used to differentiate and select male and female, and that led to wrong sexing of some fishes. This thus, increased number of sexes in some tanks by 1 or 2. So instead of having 5♂:5♀, some tanks had 4 or 3♂:6 or 7♀, and vice versa (6 or 7♂:4 or 3♀).

-In replicate tanks where 5-8 fishes died it was difficult to get a specimen at the 60-day sampling for analysis.

Experiment II

-Available fish weighing 5 -10g appeared old, because after 3days in glass tanks some started breeding (i.e. a fish weighing 8g had eggs in the mouth so had to be removed)

-Most of the fish were stunted, produced in November-December 2010, and due to limited space available were over crowded (Fry nursed at high densities for considerable periods of time, prior to stocking may be stunted, but sexually matured and will thus spawn at smaller size, Mair & Little, 1991). This is the case of fish in grey system being reared, which I had to use for the experiment, thus the long stocking period.

-Fishes showing signs of maturation and spawning behavior had to be replaced with sexually immature ones, hence the wide size range, 2-8g.

-It took 20days to get sexually immature fish to stock the 55 tanks

3 CHALLENGES

Some Challenges on the construction

The whole layout of the experimental unit was constructed between March and June 2010. The materials (glass tanks, steel stands and blue tanks) were parts of an old aquaria system in Block 2 of DA offices. It was dismantled and sent to a greenhouse attached to Block 3 (DA-Buildings) in September 2009. The layout and arrangement of the platforms commenced in October 2009, however, actual construction began in April 2009 and completed in July 2010. Water pumps and heaters were installed and tested in August 2010. The whole experimental unit became fully operational in September 2010, and actual experiment commencing in October 2010. The major challenges encountered in the construction include:

- slow pace in ordering (by technical manager of the DA-WEF) and delivery of materials by suppliers e.g. it took over 2-months for PVC pipes to be delivered (order placed in September 2009 arrived in December 2009)
- non-committal attitude of the technical manager who was to provide technical guidance.
- delay in delivery of components constructed by external contractors e.g. the carrier of inlet-pipes, parts of broken glass tanks
- delay in installation of water pump and heaters
- continuous alterations in the design, particularly, the filtration component

The whole system was built based on my own design adapted from existing recirculating systems on the farm and construction done alone.

The experiment could not commence in February 2011 as was planned in 2010, due to repairs of the experimental unit described above. The main challenge was unavailability of fingerlings in right size 2g. Available fish weighing 5-10g were old, after 3days in glass tanks some started breeding. Capacity of available facilities do not permit production of my enough fingerlings, hence have to take samples from the general pool of fingerlings produced in the farm.

Most were stunted, produced in November-December 2010 (fry nursed at high densities for considerable periods of time, prior to stocking may be stunted, but sexually matured and will thus spawn at smaller size, Mair & Little, 1991). This is the case of fish in grey system (Welgevallen Experimental Farm DA-SU), which I had to use for the experiment, thus taking a long stocking period. Fishes showing signs of maturation and spawning behavior had to be replaced with sexually immature ones, hence the wide size range, 2-8g. It took 20days to get sexually immature fish to stock the 60tanks.

Fish Storage

Samples of fish kept in Department of Genetics central cold room from the first experiment showed signs of deterioration when I went for them in October 2011 for the HPLC analysis. I was informed by the manager of the cold room that the system broke down twice within the year 2011 and they could not transfer my samples to other department cold rooms. The study wanted to do analysis on the head, flesh and internal organs. However, due to the level of deterioration of internal organs, particularly, the gonads could not be used for the analysis that was done.