#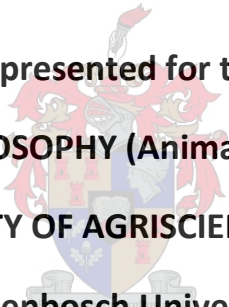 Effect of Pawpaw (*Carica papaya*) seed meal on the reproductive, endocrine and immune system of Mozambique tilapia (*Oreochromis mossambicus*)

VICTOR OKONKWO OMEJE

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March 2016

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 sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: March 2016

i

Acknowledgements

To God be the glory

Dedication

I dedicate this thesis to the three women that matter most in my life:

My mother Mrs. Virginia Oyibo Omeje

My wife Engr (Mrs) Celestina Chinyere Omeje,

and

My daughter Favour Afoma Omeje

Summary

Aquaculture, the farming of aquatic animals and plants, has the potential to solve the problems of dwindling catches from artisanal fisheries as a result of overfishing and habitat degradation. Tilapia species is one of the most cultured food fish worldwide, second only to carp. In Sub-Saharan Africa, which is in dire need of food security, tilapia has the potential to be a cheap source of protein, which through its cultivation, can contribute to poverty alleviation among the rural poor communities. Tilapia breeds effortlessly in captivity, with this attribute which is considered as the "Achilles heel" of the species, because it predisposes pond systems to overcrowding and low weight at harvest. Efforts to mitigate this shortcoming include mono-sex culture of all-males using exogenous hormone to reverse the sex of sexually undifferentiated fish. This is premised on the fact that improvement in the growth by mono-sex culture will lead to shortened production times and a more uniform weight at harvest, which will ultimately benefit the producers. However, the use of exogenous hormones in aquaculture has recently raised concerns about the effect on farm workers, consumers and on the environment. Recently research has focused on the use of substances of plants origin which mimic the action of hormones as a potential approach to achieve sex reversal in fish. Pawpaw (*Carica papaya*) seed meal (PSM) contains phytochemicals that hold great promise as a sex reversal and a reproductive inhibition agent in aquaculture. The objective of this study was to determine the optimum inclusion levels of PSM that will produce the highest percentage of male brood when included in the diet of sexually undifferentiated Mozambique tilapia (*Oreochromis mossambicus*; OM) fry of approximately one to two weeks old. Furthermore the study investigated the effects of the PSM on the reproductive hormone profile, haematological and serum biochemical parameters, and gonad and liver integrity of pre-vitellogenic OM. At an inclusion level of 10 g/kg of basal diet, PSM was able to skew the sex ratio in favour of males (60% males to 40% females). The proportion of males increased with an increasing dosage of PSM, with the maximum masculinization achieved at an inclusion level of 20 g/kg BD, resulting in 77.8% males produced. When the masculinization success was compared in terms of the duration of the feeding regimes of one and four months, no significant differences were observed in terms of the number of males produced. The inclusion of PSM did not affect the growth and survival rates, neither did it affect the Fulton's condition factor of the treated fish. It was found that the PSM investigated lowered the level of plasma 17β- estradiol in female fish but had no effect on the level of the same hormone in males. The plasma levels of 11-ketotestosterone was not affected in both genders. The gonad weight and gonado-somatic index of the male fish were not affected by treatment with PSM, while the gonad weight, GSI, fecundity and egg diameter of the treated females were lower than those of the control. Some of the changes induced returned to normal on cessation of treatment suggesting a reversible reproductive inhibition by PSM. Haematological and biochemical profiles of different treatment groups did not differ throughout the course of the investigation. Liver weight and hepato-somatic index of the treated fish were comparable to those of the control. Histological observations showed minor alterations in the architecture of the liver, with degeneration and vacuolization of hepatocytes in less than 10% of the members in the group fed 30 g of PSM /kg of basal diet for 60 days. However this was not noticed among the group fed 30 g of PSM/kg of basal diet for 30 days, suggesting a possible reversibility of the lesion on withdrawal of treatment. The current research has clearly demonstrated the potential of PSM as a fertility inhibitor and sex reversal agent in OM, with potential application in rural fish farming and feed manufacturing industries. The possibility

exist that some of the findings can be adapted to be applicable in other tilapia species like *O. niloticus* or *Sarotheridon galilaeus* which together with *O. mossambicus* constitute the most cultured species in Sub-Saharan Africa.

Opsomming

Akwakultuur, die boerdery van akwatiese diere en plante, het die potensiaal om die probleme van die kwynende vangste van ambagsvissers, wat die gevolg is van oorbenutting van visbronne en habitat vernietiging, op te los. Tilapia spesies is een van die mees gewilde voedselvis spesies wêreldwyd, naas karp. In Sub-Sahara Afrika, wat 'n dringende behoefte aan voedselsekuriteit ervaar, verteenwoordig tilapia 'n goedkoop bron van proteïen en wat deur die produksie daarvan, die potensiaal het om by te dra om armoede verligting in landelike gemeenskappe. Tilapia spesies teel moeiteloos in aanhouding, maar hierdie eienskap word ook as die "Achilleshiel" van die spesie beskou, want dit lei tot oorbevolking van damme en 'n lae eindgewig wanneer die vis geoes word. Pogings om hierdie tekortkoming aan te spreek sluit die produksie van enkelgeslag groepe, waar die geslagsdifferensiasie van ongedifferensieerde vingerlinge deur middel van die toediening van eksogene hormone gemanipuleer word. Die beginsel is gebaseer op die feit dat die verbetering in die groei in enkelgeslag groepe sal lei tot 'n verkorte produksietydperk en 'n meer eenvormige gewig by die oes, wat uiteindelik tot voordeel van die produsente sal wees. Die gebruik van eksogene hormone in akwakultuur het egter onlangs kommer veroorsaak as gevolg van die potensiële invloed op plaaswerkers, verbruikers en die omgewing. Onlangse navorsing het gefokus op die gebruik van middels van plantoorsprong wat in die plek van eksogene hormone gebruik kan word om die manipulasie van geslag en die uiteindelike produksie van enkelgeslag groepe, moontlik te maak. Papaja (*Carica papaya*) meel gemaak van papaja pitte (PSM) bevat fitochemikalieë wat die potensiaal het om die geslag van ongedifferensieerde vingerlinge te manipuleer om enkelgeslag groepe te produseer en so dus as 'n reproduksieonderdrukkende stof in akwakultuur sisteme gebruik te word. Die doel van hierdie studie was om die optimale insluitingsvlak van PSM, wanneer dit as deel van die dieet van ongedifferensieerde Mosambiek tilapia (*Oreochromis mossambicus*; OM) ingesluit word, wat die hoogste persentasie manlike vis tot gevolg sal hê. Verder het die studie die invloed van die PSM op die reproduksiehormoon profiel, hematologiese - en serum biochemiese parameters, asook geslagsklier- en lewer integriteit van pre-vitellogeniese OM bepaal.

Die PSM het by 'n insluitingsvlak van 10 g PSM/kg basale dieet (BD) die geslagsverhouding ten gunste van manlike vis verander (d.i. 60% manlik vs. 40% vroulik). Die verhouding van manlike vis het toegeneem met 'n toenemende dosis PSM, met die maksimum vermanliking van 77.8% wat met 'n insluitingsvlak van 20 g PSM/kg BD verkry is. Wanneer die vermanliking sukses vergelyk is in terme van die duur van die behandelingstydperk van 1 en 4 maande onderskeidelik, is geen betekenisvolle verskille waargeneem in terme van die persentasie vermanliking nie. Die insluiting van PSM het geen invloed op die groei en oorlewing van OM gehad nie en dit het ook geen invloed op die Fulton kondisiefaktor van die behandelde vis gehad nie. Daar is gevind dat die PSM die plasmavlak van 17β-oestradiol in vroulike vis verlaag het, maar dit het geen effek op dié hormoon se vlakke in die manlike vis gehad nie. Geen invloed van die PSM is op die plasmavlakke van 11-ketotestosteroon in beide geslagte waargeneem nie. Die gonade gewig en die gonado-somatiese indeks (GSI) van die manlike vis is nie deur die PSM behandeling beïnvloed nie, terwyl die gonade gewig, GSI, vrugbaarheid en eier deursneë van die wyfie visse laer as dié van die wyfies in die kontrole groep was. Met staking van die PSM behandeling is daar 'n omkering van die inhibering van reproduksie wat deur die PSM veroorsaak is, waargeneem. Die hematologiese en biochemiese profiel van die onderskeie behandelingsgroepe het nie betekenisvol verskil deur die verloop van die ondersoek nie. Lewergewig en

die hepato-somatiese indeks van die behandelde vis was vergelykbaar met dié van die kontrole groep. Histologiese waarnemings het klein veranderinge in die argitektuur van die lewer, d.i. degenerasie van en die vorming van vakuole in hepatosiete in minder as 10% van die visse wat 30 g PSM/kg BD vir 60 dae gevoer is. Hierdie defekte is egter nie waargeneem by visse wat 30g PSM/kg BD vir 30 dae ontvang het nie, wat dus dui op 'n moontlike omkeerbaarheid van die lewerskade met onttrekking van die behandeling. Die studie het duidelik getoon dat PSM effektief gebruik kan word om reproduksie te onderdruk as ook om vermanliking van groepe moontlik te maak, met hierdie twee bevindinge wat praktiese toepassing in landelike visboerderysisteme en voervervaardigingsbedrywe het. Die moontlikheid bestaan dat sommige van die bevindinge aangepas kan word vir ander tilapia spesies soos *O. niloticus* of *Sarotheridon galilaeus*, wat saam met *O. mossambicus* die volopste in Sub-Sahara Afrika.

List of tables

List of figures

List of plates

Table of contents

Chapter 1

General introduction

Aquaculture, defined as the rearing or farming of aquatic organisms, has been recognized as a reliable alternative for increasing food production for the ever increasing human population of especially the developing countries. Globally, aquaculture is considered as the most rapidly growing animal food production sector, with an annual growth of 5.8% and contributing 70.5 million tonnes in 2013 (FAO, 2014). Aquaculture also plays a major role as a provider of direct and indirect employment to rural poor communities, thereby contributing towards poverty alleviation.

The family Cichlids is a highly diversified group of fish and include amongst others tilapia, which is the most cultured fish species in Sub-Saharan Africa. Because of its widespread occurrence, growth on natural grazing or formulated feeds with no constraint for seed production, disease resistance and high consumer acceptability, cichlid species are extremely suitable as food fish species that can potentially alleviate food security in rural communities in Sub-Saharan Africa (Little, 1998). Tilapia species are hardy and highly prolific, and breeds effortlessly in captivity, unlike species such as *Clarias gariepinus, C. angullaris, Heterobranchus bidosarlis*, and *H. longifillis* (Reed *et al*., 1967). To ensure prolific breeding of the latter species in captivity, the use of exogenous hormones is required to induce spawning in captivity.

Mozambique tilapia *(Oreochromis mossambicus*) is one of the most prolific members of the tribe Tilapiini (Trewavas, 1982) and reaches sexual maturity at a size of approximately 15 g (Popma & Lovshin, 1995). In tilapia aquaculture, the consequence of early maturity on the overall reproductive performance is of great importance in culture systems. Precocious maturation and indiscriminate breeding result in overcrowding of ponds and stunted growth, which in turn results in a low percentage of marketable size fish obtained in mixed sex culture systems (Toguyeni *et al.*, 2002). Implementing all-male tilapia production systems offers a potential means to overcome the problems associated with overstocking and stunted growth, and is based on the premise that male tilapia grows faster and bigger than females (Beardmore *et al*., 2001). The techniques employed in tilapia production systems to achieve all-male populations range from manual separation of sexes, genetic/chromosomal manipulations (super male tilapia), environmental manipulation (such as heat shock), and endocrine manipulation of gender (including administration of 17α-methyltestosterone) (Abucay *et al.*, 1999; Beardmore *et al*., 2001; Desprez *et al.*, 2003; Abad *et al.*, 2007). The endocrine manipulation of fish

gender is the most effective method to create all-male tilapia populations, but the use of the meat is prohibited in many countries since hormone residues remaining in the meat may adversely affect human health (Curtis *et al.*, 1991; Khalil *et al.*, 2011). Alternative treatment strategies that include phytochemicals such as phytoestrogens that mimic the action of the endogenous fish hormones, is considered as a suitable substitute for 17α-methyltestosterone to produce all-male tilapia populations (Ampofo-Yeboah, 2013). In the study of Ampofo-Yeboah (2013), sexually undifferentiated *O. mossambicus* received diets containing 15g of pawpaw seed powder per kg of basal diet, and this inclusion level resulted in 65% males. There is the need to quantify the optimal inclusion level and duration of treatment to establish the dosage that will provide the maximum percentage of sex reversal, and to assess the influence of the treatment on the overall health status of the fish, as measured by the immune status and liver function.

Similar to other animals, the majority of physiological processes in fish (i.e. reproduction, digestion, metabolism, growth and development) are regulated by one or more endocrine glands. Gonadotropin releasing hormone (GnRH) produced in the preoptic area of the hypothalamus stimulates the anterior pituitary gland to produce and release the gonadotropin hormones: follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Devlin & Nagahama, 2002). The gonadotropin hormones act on the gonads (ovary and testes) to stimulate gametogenesis and the eventual production of the reproductive steroids, 17β-estradiol and testosterone (Yaron & Levavi-Sivan, 2011). The reproductive steroids stimulate gonadal maturation, ovogenesis and ovulation in female fish, and spermatogenesis and spermiation in male fish. The production and release of gonadotrophic hormones, in particular LH, by the pituitary gland are inhibited by the neurotransmitter dopamine (Zohar *et al.*, 2010). The physiological principle behind the dopamine inhibition of the release of LH can be applied in aquaculture to control the maturation of the gonads and thus the reproductive potential of highly prolific species such as tilapia. Understanding the steroid hormonal profile of a particular fish can facilitate the development of methods of controlling or inducing its reproduction in a pond system.

Gonadal development and fecundity of fish are affected by certain endocrine disrupting chemicals. According to Casanova-Nakayama *et al.* (2011), endocrine disrupting compounds exert their biological activity either by interacting with endogenous hormone receptors or by disturbing endogenous hormone metabolism. Studies have shown that many antifertility compounds contained in parts of plants have the potential to disrupt reproductive endocrine pathways (Biswas *et al*., 2002; Huang & Chen, 2004), similar to the influence of dopamine. Makkar *et al*. (2007) reported masculinization of tilapia larvae fed on a diet containing 700 ppm of quillaja saponins. Crude extracts of different parts of Moringa (*Moringa oleifera*) and pawpaw (*Carica papaya*)

plants have been used for the partial reduction or complete suppression of reproduction in fish (Mousa *et al.*, 2008; Hossam & Wafaa, 2011). Ekanem & Okoronkwo (2003) used an inclusion level of 9.8 g/kg of pawpaw seed meal per day to induce permanent sterility, and 4.9 g/kg of pawpaw seed meal per day to induce reversible infertility in male Nile tilapia (*Oreochromis niloticus*). Seeds of pawpaw (*C. papaya*) contain a number of chemical compounds, some of which include fatty acids, crude protein, crude fiber, papaya oil, carpaine, oleanolic glucoside, benzyl isothiocyanate, benzyl thiourea, hentriacontane, β-sitosterol, caricin and an enzyme myrosin (Tang, 1973; Marfo *et al.*, 1986; Krishna *et al*., 2008). It is believed that the benzyl isothiocyanate and oleanolic glucoside (triterpene acid) component of pawpaw seeds are responsible for the antifertility properties of the plant (Wilson *et al.*, 2002; Jegede& Fagbenro, 2008; Krishna *et al*., 2008; Ampofo-Yeboah, 2013). However, an attempt to quantify triterpene acids (oleanolic and ursolic acids) components of the pawpaw seed using high performance liquid chromatography – mass spectrometer (HPLC-MS) failed to recover the triterpens (Ampofo-Yeboah, 2013).

According to Harris & Bird (2000), the immune and neuroendocrine systems are intimately linked and bi-directional communication between the two systems is essential for the maintenance of homeostasis. The immune system is important for the survival of fish, and immunoglobulins play an integral role in the mobilization of an immune response against pathogenic organisms (Uribe *et al.*, 2011). Immuno-stimulants are compounds that improve the innate defence mechanisms of fish, and are sometimes employed in aquaculture enterprises as a preventive measure against many microbial infections. Casanova-Nakayama *et al.* (2011) stated that certain chemical compounds that occur in plants can affect a variety of physiological systems other than the reproductive system in a beneficial way. Some phytoestrogens are believed to be immuno-competent, and according to Rayes (2013), Moringa plant has the potential to boost the immune system of shrimp (*Penaeus indicus*). However, some plant phytochemicals have the potential to adversely affect the physiological processes in the animals including fish (Pathak *et al.*, 2000; Clotfelter & Rodriguez, 2006; Ayotunde & Ofem, 2008). Haematological parameters seems to be the most reliable indicator of alteration in physiological processes in fish, and it is believed that the effect of certain phytochemicals can be determined by the assessment of haematological and blood serum parameters (Akinrotimi *et al.*, 2012). The main functions of the liver include the assimilation of nutrients and detoxification of toxins, and these functions makes it a target for attack by the toxicants (Ighwela *et al*., 2014). The ratio of liver weight to body weight termed the hepatosomatic index (HSI) is valuable in the study of the effect of xenobiotic or treatment on fish species. Evaluation of the HSI and histological changes in the architecture of the liver makes it possible to identify pathological conditions such as atrophy, hypertrophy or hyperplasia associated with disease conditions or the effect of toxicants (Al-Ghais, 2013).

The purpose of the study was to determine the maximum inclusion level of pawpaw (*C. papaya*) seed meal in the diet of *O. mossambicus* that will result in the highest degree of masculinization of Mozambique tilapia in pond systems to ultimately inhibit precocious maturation and indiscriminate spawning in pre-vitellogenic fish without endangering the health and wellbeing of the fish.

Specific aspects that were investigated in the study included the following:

1. Determination of the maximum inclusion level of Pawpaw (*C. papaya*) seed meal as part of a commercially available tilapia diet to ensure effective masculinization of populations, without affecting the growth and survival of Mozambique tilapia (*O. mossambicus*).

2. The effect of the Pawpaw (*C. papaya)* seed meal on the reproductive hormone profile, gonado-somatic index, egg diameter, and fecundity of *O. mossambicus.*

3. The effect of Pawpaw (*C. papaya)* seed meal on the respective serum biochemical parameters, i.e. blood glucose, cholesterol, total protein, albumin and globulin levels.

4. The effect of Pawpaw (*C. papaya)* seed meal on the haematological parameters of the fish, i.e. red blood cell counts, haemoglobin, packed cell volume (haematocrit), mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, red cell distribution width, thrombocytes (platelets), white blood cell counts, neutrophils, lymphocytes, monocytes, eosinophils, basophils

5. The effect of Pawpaw (*C. papaya)* seed meal on the architectural integrity of the liver and the gonadal tissues.

By improving our understanding of the use of pawpaw seed powder as part of basal diets of Mozambique tilapia to skew the gender of production populations, we will be able to formulate a treatment protocol for tilapia production systems that can be used by rural communities to farm profitably with tilapia, thus addressing food and household security. It will also assist in minimizing the negative effect of exogenous hormones on the environment, thereby ensuring the production of a sustainable and safe food source, without compromising the environment and human health.

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Chapter 2

Literature Review

2.1 Aquaculture as an international food source

The farming or culture of aquatic animals and plants in a controlled system is referred to as aquaculture. The farming of fish is believed to have originated in Asia, and particularly in China, as far back as 1100 BC, when common carp were raised in freshwater ponds for food (Bondad-Reantaso *et al.*, 2005). In the food producing sector aquaculture is internationally acknowledged as the fastest growing sector, with Asian countries alone contributing more than 90% to this production (Bondad-Reantaso *et al.*, 2005).

Contribution of aquaculture to human nutrition is considerable, with world aquaculture production of food fish estimated at 66.5 million tonnes in 2012 (Pullin & Neal, 1984; FAO, 2013). At present, the number of finfish and shellfish species that are cultured for consumption is approximately 220 species, and include amongst others giant clams, mussels, salmon, carps, and tilapia (Naylor *et al.*, 2000). The increased demand for increased fish production through aquaculture is compelling, since the consumption of aquatic food is increasing whereas the catches from the wild stock is dwindling (De Silva, 2003). As the production from capture fisheries are dwindling, the world population is rising astronomically, reaching numbers as high as 6.63 billion people in 2007 (Diana, 2009). The last 15 years alone witnessed a two-fold increase in farmed fin and shellfish production in weight and value globally (Naylor *et al.*, 2000). According to the Food and Agricultural Organization (FAO) (2013), food fish production from aquaculture increased from 59 million tonnes in 2009 to about 62.7 million tonnes, which represent about a 6.2% growth.

Aquaculture has a positive impact on food security, household income and poverty alleviation especially among rural, poor communities (Ahmed & Lorica, 2002). Commercial fish farming especially in low income food deficit countries (LIFDC for example Benin, Ghana, Sri Lanka and Bangladesh), have been recognized as an important tool to increase food fish availability and accessibility, and also income generation through employment (Hishamunda & Ridler, 2006). Although aquaculture practices in developing countries are subsistence in nature and may not provide substantial employment to the teeming population, its impact in poverty alleviation cannot be ignored. Fish contributes over 25% of total animal protein intake worldwide especially in low income and developing countries. It is a good source of vitamins especially A, D, E and B- complex vitamins and also omega-3 fatty acids (Bondad-Reantaso *et al.*, 2005).

China as the world leader in aquaculture production together with other Asian countries like India, Philippines and Indonesia, contributes about 89% of the global total cultured fish production (Anamarija & Hershner, 2003). Data from the Fisheries and Aquaculture Department of FAO on the year 2011 aquaculture production shows that Asia remains the world leader in terms of total world production, producing 89% of all aquaculture products consumed. Sub-Saharan Africa produces approximately 2%. Out of the 20 world leaders in aquaculture production listed in Global Aquaculture Production Statistics for the year 2011, Egypt was the only African country that was included in this list (FAO, 2013).

Brummett *et al*. (2008) extensively reviewed the development of aquaculture in Sub-Saharan Africa, and concluded that despite the enormous capital investment in research and development, the aquaculture sector is still lacking in meeting the increasing demands for food fish production. Despite the slow pace of development of aquaculture in Sub- Saharan Africa, the sector have contributed through environmentally friendly and easily adaptable farming systems that enable the production of food fish by especially rural communities in Sub-Saharan Africa. In Africa, Egypt is perhaps the first country to venture into the culture of fish through using freshwater ponds for fish production activities (Bondad-Reantaso *et al.*, 2005). Incidentally tilapia was the first cultured fish in Egypt, about 2500 years ago, and today Egypt is considered as the biggest producer of food fish, contributing 72% of Africa's production (Brummett & Williams, 2000; FAO, 2010). According to the FAO (2010), Nigeria and Uganda is the second and third biggest producers, producing 16% and 7% respectively, of Africa's production. Together these three countries produce about 94% of the entire continent's production as indicated in Table 2.1. However, current data from FAO World Fisheries and Aquaculture indicated that African countries production of food fish in 2012 was 5.86 million tonnes (FAO, 2014)

Table 2.1 Aquaculture production in African countries during 2010 (adapted from FAO, 2010).

Ranking	Country	Production (tonnes)	Percentage
1	Egypt	919 585	71.51
2	Nigeria	200 535	15.59
3	Uganda	95 000	7.39
4	Kenya	12 154	0.95
5	Zambia	10 290	0.80
6	Ghana	10 200	0.79
7	Madagascar	6 886	0.54
8	Tunisia	5 424	0.42
9	Malawi	3 163	0.25
10	South Africa	3 133	0.24
11	Democratic Republic of the Congo	2 970	0.23
12	Zimbabwe	2 702	0.21
13	Sudan	2 200	0.17
14	Mali	2 083	0.16
15	Algeria	1 759	0.14
16	Cote d'Ivoire	1 700	0.13
17	Rocco	1 522	0.12
18	Mozambique	864	0.07
19	Cameroon	628	0.06
20	Rwanda	628	0.05
21	Others	2 339	0.18
	TOTAL PRODUCTION	**1 285 972**	**100**

2.2 Tilapia as a food fish

Tilapia refers to several freshwater species that belong to the family Cichlidae, and comprises of about 80 species of fish. Tilapia originates from Africa, and because of its easy adaptability to various environmental conditions, has been introduced to many countries. The low cost of production combined with the fact that tilapia is widely accepted by consumers as a food fish, promotes its culture worldwide (Siddiqui & Al-harbi, 1995; De la Fuente

et al., 1999; Rad *et al.*, 2006; Shalloof & Salama, 2008; El-Kashief *et al.*, 2013). Statistics generated by the Food and Agriculture Organization (FAO) has indicated that tilapia production in 2001 amounted to 1.5 million tonnes (Figure 2.1), with an expectation to increase progressively over the years (FAO, 2002). World tilapia production for the year 2012 exceeded 4.51 million metric tonnes (FAO, 2014). When Africa is compared to other countries that farm with tilapia, e.g. countries like China, the Philippines, Taiwan, Thailand, USA and Belgium, Africa is outperformed in terms of production, with these countries collectively producing more than 850 000 tonnes annually (Coward & Bromage, 2000). Tilapia production in 2010 exceeded 3.2 million metric tons, exceeding the production of both salmon and catfish. Although second to carp in terms of production, it is expected that in the near future with the greater acceptance and wider distribution enjoyed by tilapia, it will become the most important aquaculture species.
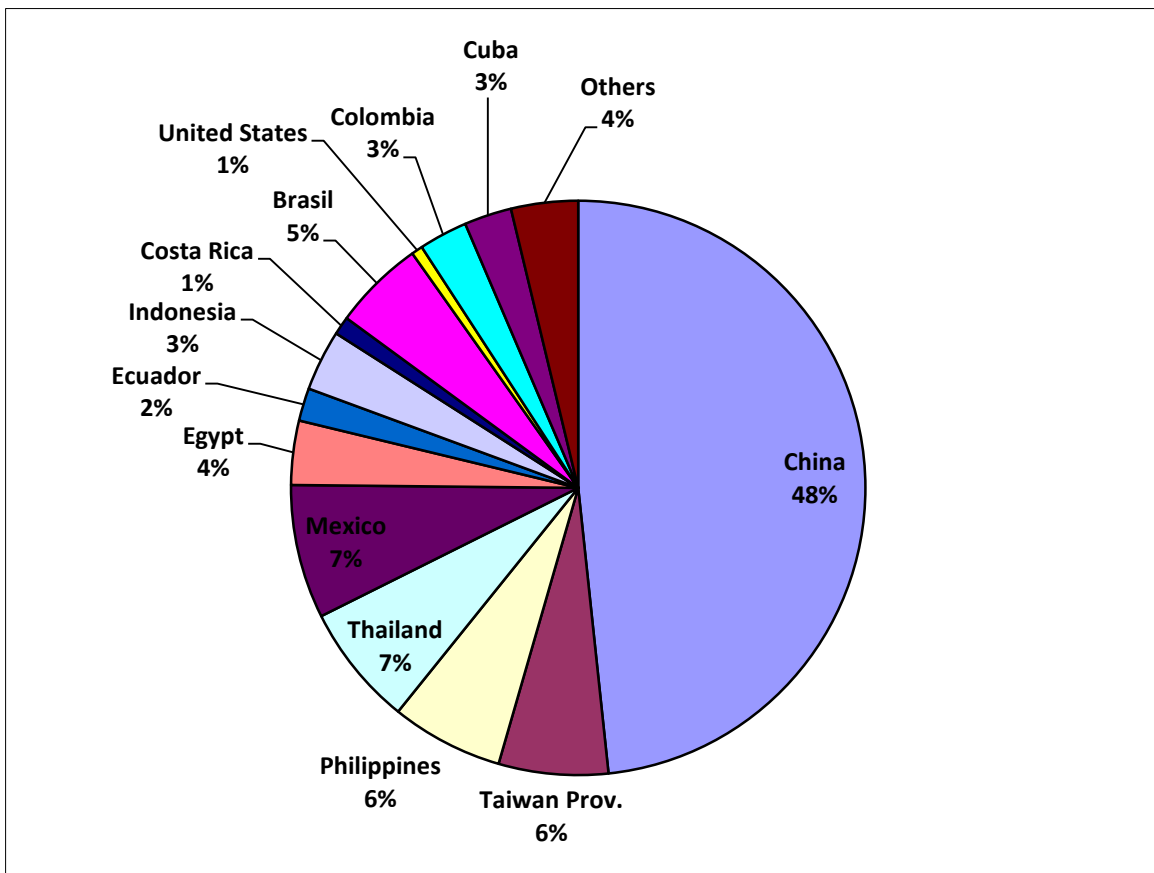
Figure 2.1 World Tilapia Production of 1.5 million tonnes in 2001 (adapted from FAO Fisheries and Aquaculture Department, 2002).

Even though the group Tilapia is made up of more than 80 species of fish, only 8 or 9 species are considered important in terms of production (Coward & Bromage, 2000). Tilapias are considered as a good source of food fish especially for the low income food deficit countries with three species in the genus *Oreochromis* (*O. niloticus,*

O. mossambicus, and O. aureus), two species in the genus *Tilapia* (*T. rendalli* and *T. zilli*), and one species in the genus *Sarotheridon* (*S. galilaeus*) being the most cultivated of the family (Siddiqui & Al-Harbi, 1995; El-Kashief *et al.*, 2013). The family Cichlidae has three main genera *Oreochromis*, *Tilapia* and *Sarotherodon.* Previously all tilapia fish were grouped together under the genus Tilapia, but recently two other groups were established based on the parental care investment of the particular species. The genus *Oreochromis* exhibit maternal mouth brooding, the genus *Sarotherodon* is characterized according to the mouth brooding behaviour exhibited by both parents, and the *Tilapia* genus are classified as substrate spawners (Coward & Bromage, 2000; Specker & Kishida, 2000; Fishelson & Bresler, 2002). Fecundity of tilapia varies inversely with the parental care exhibited by the species, which means that the more the parental care is invested, the lower the fecundity of the species will be. The fecundity of species as *O. mossambicus*, which is a mouth brooder, is as low as 350, compared to substrate spawners such as *Tilapia zilli*, which has a fecundity of 12 000 eggs (Coward & Bromage, 2000). Members of the Tilapia family exhibit various degrees of parental care for their offspring. The genus *Oreochromis* orally incubates eggs and larvae, and the mouth brooding practice continues up to juvenile stage where the female at any threat or danger, takes the young into her mouth for safety (Tacon *et al.*, 1996).

Oreochromis is the most diverse of the genera, and contain amongst others the species *O. niloticus, O. mossambicus, and O. aureus*. *Oreochromis mossambicus* (OM) is one of the most important members of the tilapia family, second only to *O. niloticus* in terms of production output (Campos-Ramos *et al.*, 2003). The species is extremely tolerant of high levels of salinity, which makes it a good candidate for culture in marine and brackish waters (Ron *et al.*, 1995; Kamal & Mair, 2005). The culture characteristics that make OM a preferred species to farm with include their easy growth on natural grazing or formulated feeds, with no constraint for seed production, disease resistance and high consumer acceptability. *Oreochromis mossambicus* are hardy and prolific, and breeds effortlessly in captivity. During breeding season, the males dig nests (also known as pits) in shallow water, and establish a territory which it defends aggressively against other males. They attract reproductively ready (ripe) females to the nest and after spawning, the females takes up the eggs and milt in her mouth for incubation and subsequently brooding, with brooding that lasts between 20 – 22 days (Oliveira & Almada, 1996). As the female leaves the nest with the fertilized eggs in her mouth, the males continue to attract other females for another round of spawning and fertilization. As the swim-up fry start to mature they start to leave the mother during which time she guard them from predators and on any sign of danger, she will take the brood into her mouth. The fry swim in schools with their mother whenever they are released for feeding until when they reach the size at which they can fend for themselves. The feeding of the females is usually interrupted during the brooding period (Specker & Kishida, 2000)

2.2.1: Reproductive physiology of tilapia

The anatomy and function of the gonads

The ova and sperm which are necessary prerequisites for the reproduction and survival of any species are produced in the ovary and testes, respectively. The testis tissue consists mainly of seminiferous tubules, which is the site of spermatogenesis. The epithelium where spermatogenesis occur, also contain the Sertoli cells that support the developing spermatogonia. The Leydig cells are located in between the seminiferous tubules, and are responsible for testosterone production. Sertoli cells provide physical support and nourishment to the germ cells whereas Leydig cells produce the sex steroids responsible for the production of male gametes and the secondary sex characteristics (Hafez *et al*., 2008). Spermatogenesis in tilapia is asynchronous with germ cells in various stages of development, which enable multiple spawning opportunities during the breeding season (Schulz *et al.*, 2010).

In the ovary, the ovarian follicles in most fish species are morphologically similar, and consist of the oocytes and the surrounding inner granulosa and outer theca cells. Germinal vesicles contained in the oocytes can be centrally or eccentric located. Zona radiate lies between the oocytes and the granulosa cells while basement membrane separates the granulosa and theca cells. The granulosa layers are enclosed within a basal lamina that physically isolates the interior of the follicles from the surrounding stroma. Adjacent to the basal lamina are several layers of endocrine cells made up of the *theca interna* and the *theca externa*. In the females, the theca and the granulosa cells are the site of steroid synthesis (Kagawa, 2013).

The ovarian follicles undergo a tightly regulated programme of growth and differentiation. The mature ovarian follicles contain a fluid-filled antrum, whereas the immature pre-antral follicles do not. The oogenesis process is a sequential one with the ultimate aim of producing large gametes replete with stored nutrient materials (Horvath, 1985). The final stages of maturation of oocytes and follicles is mediated by the sex steroid 17α, 20β dihydroxy-4-pregnen-3-one, a maturation inducing substance (MIS) produced by the follicular envelope in response to pituitary gonadotropin (Figure 2.2). Pituitary gonadotropin induces the theca cells to produce 17α hydroxy-4-pregnen-3-one, a precursor of the MIS, which is then converted in the granulosa cells to the $17\alpha,20\beta$ dihydroxy-4-pregnen-3-one. The MIS permeates the *zona radiata* to be transported to the oocytes, where it induces its maturation (Yaron & Levavi-Sivan, 2011).
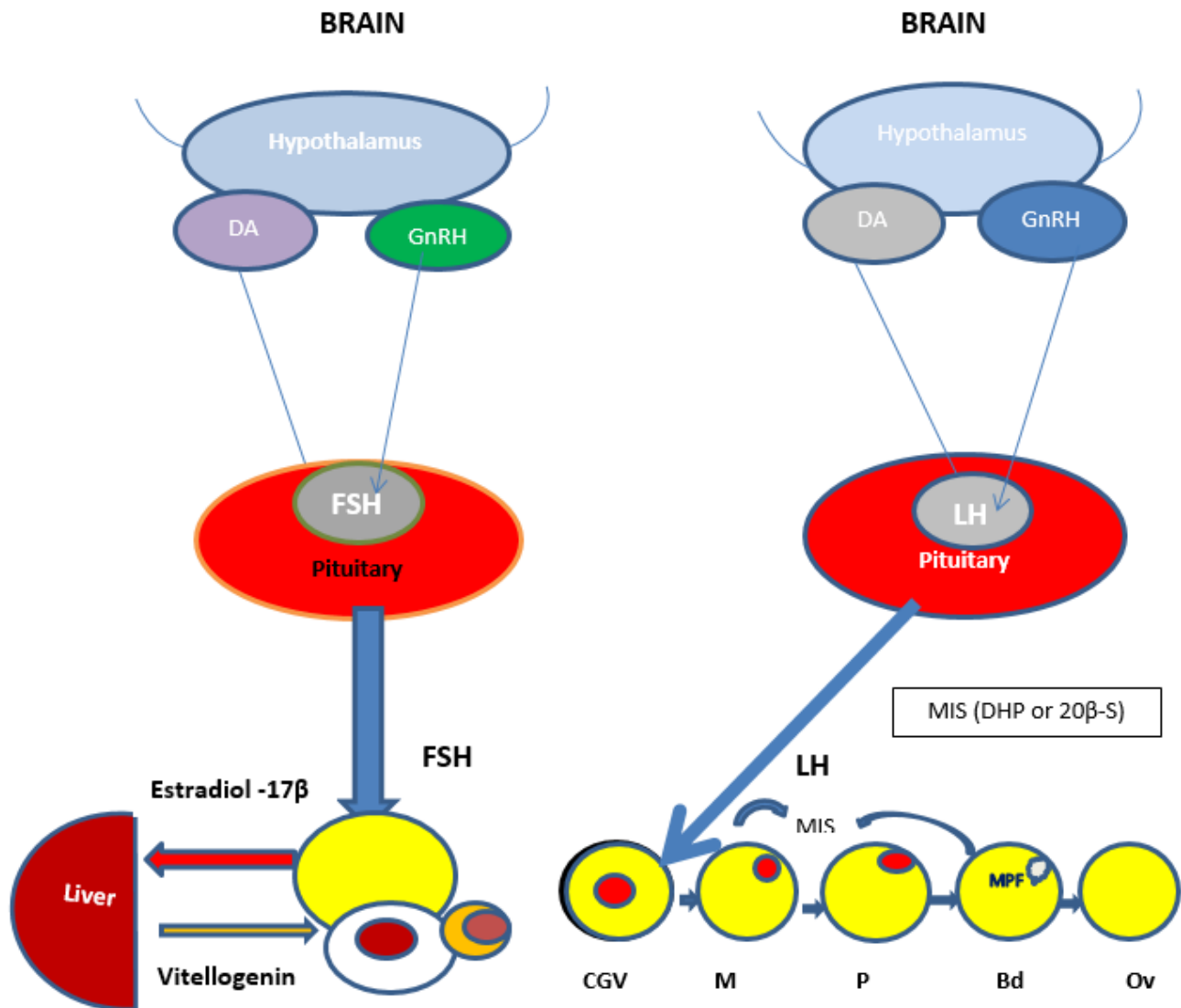
Figure 2.2 Chain of physiological events leading to Ovulation in fish: Brain-Pituitary-Gonadal Axis (BPG Axis); FSH- Follicle stimulating hormone; LH- Luteinizing hormone; MIS- maturation inducing steroids; MPF- maturation promoting factor; DHP- 17α,20β dihydroxy-4-pregnen-3-one; CGV-Central germinal vesicle; M-migrating; P- peripheral; Bd- breakdown; Ov- Ovulation (Adapted from Yaron & Levavi-Sivan, 2011).

Corticosteroid hormones may sensitize oocytes to 17α, 20β dihydroxy-4-pregen-3-one, which is vital for oocyte maturation in fish (Sundaraj & Vasal, 1976). Usually after vitellogenesis, the germ cells in fish become temporarily dormant. Breton *et al*. (1993) stated that maturation of the gonads in fish proceeds as an indirect result of a slow and steady rise in gonadotropin secretion, and ovulation and spermiation are preceded by a more marked increase in gonadotropin hormones. The release of matured oocytes from the ovarian follicle is referred to as ovulation. Ovulation is preceded by the rupture of the follicular layers aided by proteolytic enzymes, and the separation of the microvilli connecting the oocytes to the follicular cells (Blaxter, 2010). Gonadotropic hormones

14

and prostaglandins are responsible for ovulation, while the expulsion of the oocytes is made possible through the contraction of the theca cells.

Size at sexual maturation

The reproductive performance of a species is of particular interest in aquaculture, since production indices are based on the availability, and number and quality of sperm and ova produced. Since egg size increases with maternal age and size, fish maturing early and at a smaller size will produce relatively more but smaller eggs per unit body weight than larger fish (Coward & Bromage, 1999). There is a decrease in the growth rate during the time of sexual maturity, and as a consequence of this, late maturing fish are larger than their early maturing counterparts (Schreibman *et al*., 1989). Species like tilapia exhibit early sexual maturation, which result in the overcrowding of and stunted growth observed in tilapia culture systems. Tilapia species become sexually mature at a very small size, i.e. with an average live weight of 15 g, with no growth occurring after an animal has attained sexual maturity (Popma & Lovshin, 1995).

Egg size and fecundity

Maternal size is one of the factors believed to be responsible for egg size. Within a given fish species, the production of larger eggs by bigger individuals is well documented (Zonneveld & Van Zon, 1985; Bromage & Cumoranatunga, 1988). However, it is not clear whether maternal age or size is the primary factor influencing egg size. Al-Ahmad *et al*. (1988) stated that the age of brood stock does not influence the fecundity of tilapia, whereas fecundity decreases with an increase in the salinity of the culture water. Egg size is influenced by the species of fish, i.e. in the tilapia family egg size is species-specific, regardless of female age. Rana (1988) reported that when females of similar age and irrespective of size, were reared and spawned under similar conditions, mean egg size of *Oreochromis niloticus* females was found to be significantly larger than that of *Oreochromis mossambicus.*

Fecundity has been defined as the number of maturing oocytes in the ovaries prior to spawning (Bagenal & Braun 1979). The number, diameter and volume of eggs produced by female fish increases with their age, length and weight, whereas their relative fecundity which is the number of eggs spawned per kilogram body weight of the female decreases (Coward & Bromage, 1999). In fish of the same weight and total length, the size of their eggs may vary (Pena-Mendoza *et al.,* 2005). Rana (1988) was of the opinion that total fecundity is more closely related to maternal size than age. According to the author, unlike egg size, the number of eggs spawned by *Oreochromis niloticus* brooders of similar age, increased significantly with their size. Consequently in a mixed age structure, studies suggest that larger fish of the same age may lay more eggs. Maternal growth rate and factors such as

their nutritional status will also be of crucial importance in increasing egg yields for fry production. Variation in the egg size of the same individual fish indicates the multiple spawning nature of the species.

2.2.2: Hormonal regulation of ovulation and spermiation

Endocrine glands are ductless glands that produce hormones that are secreted directly into the blood stream to be transported to their targets sites (Hafez *et al*., 2008). Hormones are important regulators of processes that play a critical role in reproduction (Mc Donald & Prineda, 1989). The nervous and endocrine systems of teleost fish function synergistically to coordinate reproductive activities. Perception of environmental stimuli such as daylight and rainfall is mediated by the nervous system, and involve the relay of information from the sensory receptors to the brain. The hypothalamus, also referred to as the master gland, controls other glands and regulates the secretion of the gonadotropin hormones FSH and LH by the pituitary, which in turn act on the gonads (ovaries and testes) to stimulate and support folliculogenesis and spermatogenesis, respectively. The pituitary gland is located at the base of the brain lying immediately beneath the third ventricle in a bony cavity, the *sella turcica* (Junqueira & Carneiro, 2003).

When a favourable environment that will allow reproduction is encountered by the animal, the hypothalamus releases gonadotropin releasing hormone (GRH), which acts on the pituitary to stimulate the synthesis and secretion of follicle stimulating hormone (FSH) and luteinising hormone (LH), both of which exert a trophic effect on the ovaries and testes. The gonads are stimulated to produce the sex hormones 17β-estradiol and testosterone, which in turn play an integral role in ovigenesis and ovulation and spermiation, respectively, as well as spawning (Madu *et al*., 1984; Zohar, 1989; Mazzeo *et al.*, 2014). In males, androgens are responsible for the development of male secondary sexual characteristics, spermatogenesis and reproductive behaviour.

Androgen is synthesized from the precursor testosterone by the enzymatic activation of cytochrome $P450_{11}\beta$ (Liu *et al.*, 2000). The reproductive state of fish is reflected by the levels of testosterone and 17β-estradiol in females, and testosterone and 11-ketotestosterone in males, with higher levels reported during the reproductive period (Frisch, 2005) or prior to each spawning (Yaron *et al.*, 2001), and lower levels after spawning (Rothbard *et al*., 1987). The most important processes in the reproduction of fish are vitellogenesis and spawning (Chabbi & Ganesh, 2012). Vitellogenin, synthesized in the liver under the influence of ovarian 17β-estradiol, when incorporated into the oocytes forms the yolk (Coward & Bromage, 2000).

2.2.3: Endocrine control of reproduction

Homeostasis in fish as well as in other animals is modulated by endocrine and exocrine glands. The product of exocrine glands is carried by ducts to be excreted from the body, whereas the end product of endocrine glands is transported via the circulatory system, which carries the chemical messengers to their target organs. Many physiological processes in the animal including reproduction, growth and development, are regulated by endocrine glands.

a)   Hypothalamus – Pituitary gland – Gonadal pathways

The initiation and maintenance of gonad function is tightly regulated by the hypothalamic-pituitary-gonad (HPG) axis. Gonadotropic hormones releasing hormone (GnRH) produced in the pre-optic area of the hypothalamus are transported via the vascular system to the pituitary gland. The GnRH stimulates the pituitary to produce and release the gonadotropin hormones (GTH). Two types of gonadotropins; GTH I which stimulates ovarian growth, and GTH II which is responsible for the maturation of the ovary, can be found in fish (Coward & Bromage, 1999). Due to the biochemical resemblance of GTH I and GTH II to follicle stimulating hormone (FSH) and the luteinizing hormone (LH) of the higher animals respectively, these hormones are in fish referred to as FSH and LH.

In the female fish, FSH stimulates the ovary to produce 17β-estradiol and vitellogenin. In the ovary, the steroid hormones are produced in the granulosa and theca cells of both mature and developing oocytes and also in the interstitial cells (Cornish, 1998). Blood of sexually matured female fish contains vitellogenin, a yolk protein precursor, which when incorporated into the developing oocytes, forms the yolk (Takemura & Kim, 2001). Follicle stimulating hormone stimulate the Sertoli cells proliferation in the males leading to spermatogenesis, while LH acts during the later stages of gametogenesis where it stimulates gonadal maturation, ovulation and spermiation (Yousefian & Mousavi, 2011).

Studies have shown that there is a correlation between the increase in the expression of GnRH levels and the onset of reproductive development (Zohar *et al.*, 2010). Testosterone and 17β-estradiol exert both positive and negative feedback effects on the regulation of FSH and LH, and are important in the control of fish reproduction (Jamalzadeh *et al.*, 2014). Studies have shown significant higher gonadotropin levels during ovarian development and ovulation in tilapia. Similar changes in ovarian steroids especially higher levels of 17β-estradiol during the period of follicular development have been reported by Cuisset *et al.* (1994). Their study suggested that both gonadotropin and 17β-estradiol are involved in fish reproduction. However, during ovarian maturation and ovulation the level of gonadotropins rise while that of 17β-estradiol falls which suggests a negative feedback relationship between the two hormones (Zohar *et al.,* 2010). The level of circulating gonadotropin increases

during the period of early oocytes development then decreases during the period of vitellogenesis but rises again during maturation and ovulation in rainbow trout (*Onchorynchus mykiss*) (Whitehead *et al.*, 1983). Conversely, 17β-estradiol increases as the level of gonadotropin is decreasing during the period of vitellogenesis. Whitehead *et al.*, (1983) concluded that decreasing levels of 17β-estradiol towards the final stages of maturation stimulates increase in circulating gonadotropin which consequently led to final oocytes maturation and ovulation. According to Nagahama *et al.,* (1993), pituitary gonadotropins are of primary importance in triggering oocytes growth and maturation in fish. Tilapia exhibit different spawning cycles each of which last approximately 28 days in one breeding season (Yaron *et al.*, 2001).

b)   Gonadal steroid hormones

Whereas follicle stimulating hormone (FSH) and luteinizing hormone (LH) are glycoproteins, 17β-estradiol, progesterone and testosterone are steroids. Steroid hormones secreted by the ovary and testes (including the placenta and adrenal cortex in higher animals) have a chemical structure that consists of three six-membered phenanthrene rings, which are fully hydrogenated. The three rings are designated A, B and C, while D is the cyclopentane, a five membered ring (Figure 2.3).
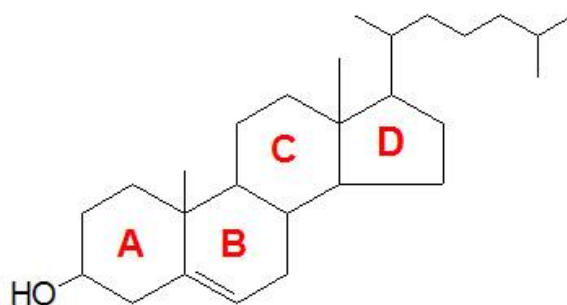
Figure 2.3 The *cyclopentanoperhydrophenanthrene* nucleus of steroid hormones (Hafez *et al*., 2008).

The number of carbon atoms in the chemical structure of a steroid hormone determines its biological action, and forms the basis for its name according to the International Union of Pure and Applied Chemistry (IUPAC). An 18-carbon steroid has estrogenic activity, a 19-carbon steroid has androgenic activity, and a 21-carbon steroid has progesterone-like properties. Cholesterol, a 27-carbon steroid, becomes pregnenolone when its side chain is cleaved. Pregnenolone is subsequently converted to progesterone, which is in turn converted to an androgen and sequentially into estrogen (Figure 2.4).

18
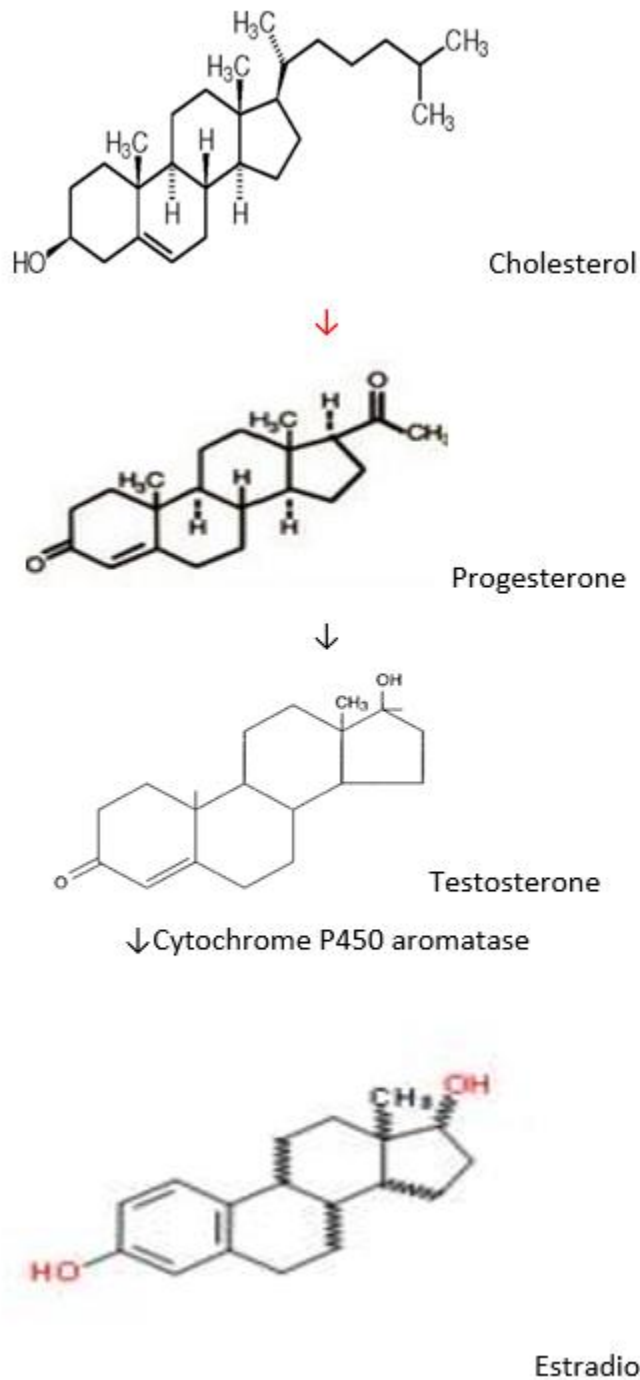
Figure 2.5 Biosynthesis of steroid hormones from cholesterol (Yaron & Levavi-Sivan, 2011).

In endocrine glands such as the ovaries and testes, several enzymes regulate the biosynthesis of the steroid hormones from cholesterol. For example, the testes synthesize androgen while the ovary synthesizes estrogen and progestin. Each of these hormones has specific target tissues with which it binds specifically to exert its effect. These target tissues or organs contain receptor proteins within its cells that bind the activating hormone. Sex steroids modulate a number of physiological responses in target tissues which are responsible for various

19

reproductive processes. They also play a role in the phenotypic expression of sex, i.e. the differentiation of male or female progeny depends on the ratio of testosterone to estrogen (Cuisset *et al.*, 1994).

Androgen

Androgens are produced in the interstitial cells of the testes in males, and also in the adrenal cortex from the precursor progesterone, which itself are converted from cholesterol. Testosterones have oxygen at the position 17 of the cyclopentanoperhydrophenanthrene nucleus and therefore are referred to as 17-ketosteroids. Androgen is the hormone that mediates the development of the secondary sexual characteristics in males and also the courtship behaviour and spermatogenesis (Hafez *et al*., 2008). According to Liu *et al.* (2000), androgen is synthesized from its precursor progesterone by an aromatase enzyme cytochrome P450. In the males, the major androgens produced include the testosterone, 11-ketotestosterone and androstenedione, the testes may also produce progesterone.

In fish testosterone is first detectable at 40 to 50 days post hatch and the level gradually increases as the fish matures until peak at the time of spermatogenesis (Nakamura & Nagahama, 1989). Production of testosterone in the gonads is catalysed by enzyme arginine vasotocin (Yaron & Levavi-Sivan, 2011). The enzyme 11β-hydroxysteroid dehydrogenase is linked to the conversion of testosterone to 11- ketotestosterone which is the major androgen present in male fish (Ribeiro *et al.*, 2012).

Estrogen

Estrogens are produced in the ovary from the precursor androgen. Estradiol is the main estrogen produced with little quantity of estrone while estriol which is the third estrogen is a metabolic by-product of estradiol/estrone (Hafez *et al*., 2008). Small quantities of estriol are also produced in the luteal phase of the reproductive cycle in females. Estrogens are converted from androgens by an enzyme cytochrome P450 aromatase (Afonso *et al*., 2001). The inhibition of the synthesis of estrogen from androgen has been employed in fish reproduction to skew the sex ratio in favour of males. Afonso *et al*. (2001) demonstrated that fadrozole, which is an aromatase inhibitor, when incorporated into the diet of *O. niloticus* at a dose of 100mg/kg of basal diet for 30 days produced 100% male individuals.

c)   Stimulatory and inhibitory factors

The functioning of the endocrine system in fish as well as other animal species is controlled by both stimulatory and inhibiting factors. Hormones produced by the pituitary gland and the gonads regulate the synthesis and release of hypothalamic hormones through positive and negative feedback mechanisms. The said feedback

mechanism can be through the interaction of the gonad, pituitary and the hypothalamus (long feedback) or the levels of pituitary gonadotropin can directly influence the synthesis and release of the hormones without the gonads being involved (short feedback) (Zohar *et al.*, 2010). Positive feedback occurs when the steroid hormones stimulate the release of gonadotropins while negative feedback occurs when they inhibit the release due probably to its high concentration in the blood. While the gonadotropin releasing hormone (GnRH) stimulates the pituitary gland to release gonadotropin hormones, dopamine inhibit the release thereof (Dufour *et al.*, 2005). Dopamine acts on GnRH neurons blocking the synthesis of the peptide or inhibiting its release from the pituitary nerve terminals (Yaron & Levavi-Sivan, 2011). Dopamine inhibition regulates the final steps of ovulation and spermiation.

2.2.4: Hormone assay

A sensitive assay of reproductive hormones is important in the study of the dynamic changes in blood levels of hormones, which will invariably lead to understanding of their roles in reproduction. The plasma levels of 11-ketotestosterone can be used to determine the gender of a fish, and Cuisset *et al.* (1991) demonstrated this in Seberian sturgeon (*Acipenser baeri*). The differentiation of the bi-potential germ cells which determines the gender of the fish, are believed to be mediated by steroid hormones. Androgens induce the differentiation of male fish, and estrogens induce feminization.

Various methods are employed for the quantitative measurement of the reproductive hormones of fish. These methods include:

- Radio-immunoassay (RIA) which is based on the theory that in the absence of unlabelled antigen or hormone, the labelled radioactive hormone has a maximal opportunity to react with a limited number of antibody binding sites. The assay has been employed in the quantitation of the serum prolactins and growth hormones in fish (Weber & Grau, 1999). Though RIA is highly specific, its major disadvantage as an assay method is that it employs radioisotopes which are highly hazardous (Rodriguez *et al*., 2000).

- High performance liquid chromatography-mass spectrophotometry (HPLC-MS) is used to separate unipolar and isomeric compounds, and it allows for the separation of multicomponent samples. The separation of the component parts of a sample using HPLC-MS is based on the assumption that compounds migrate at different rates in an electric field (Kellner *et al.*, 2004).

- Enzyme linked immune-sorbent assay (ELISA) sometimes referred to as enzyme immunoassay (EIA). The ELISA technique can be used to identify and quantify the sex steroids of fishes even when the levels are low (Rodriguez *et al*., 2000). The technique measures the amount of fish antibody produced in serum to the trinitrophenyl hapten (TNP), protein or polysaccharide antigen (Arkoosh & Kaattari, 1990). The advantages of

ELISA over other methods employed in the assay of fish hormones include that it is highly sensitive and useful in measurement of hormones of small fish where the quantity of serum from individual fish may be small (10 – 30µL) (Arkoosh & Kaattari, 1990), it is a rapid technique which involves simple techniques, and no radio isotopes are required in the protocol.

2.2.5: The role of the liver in reproduction

The liver is the organ whose functions amongst others include carbohydrate and protein metabolism, detoxification of metabolic wastes, drugs and toxins, and the synthesis of cholesterol and plasma lipoproteins such as albumin (Matos *et al.*, 2007; Taddese *et al.*, 2014). The liver plays a major role in oocyte maturation, and as a result of estrogenic stimulation, the liver produces vitellogenin which is the precursor of yolk protein, as well as choriogenin, the precursor of chorionic proteins (Yaron & Levavi-Sivan, 2011). Vitellogenin upon secretion is transported via the blood circulation to the ovary, where it is taken up by the developing oocytes to form the yolk (Rinchard & Kestemont, 2003).

Relationship between reproductive and immune systems

In higher animals, many reproductive tissues respond to lipids and proteins produced by immune cells, and this indicates an interaction of immune system in reproductive mechanisms (Seamark *et al.*, 1992). Gonadotropin releasing hormone plays an important role in the interaction between the neuroendocrine and immune systems, while the sex steroids control the development and functioning of the hypothalamo- pituitary and immune systems (Marchetti *et al.*, 1998; Zakharova *et al.*, 2005). Likewise, immune system produce mediators that participate in development of the reproductive system in animals (Goya *et al.*, 2004). A dysregulation of this interaction can lead to reproductive failure.

The liver is also one of several organs that participate in haematopoiesis in fish (Arnold, 2009). Toxic substances that can affect the liver thus also have the potential to impact negatively on the haematological parameters of fish. Blood participates directly or indirectly in almost all biochemical processes in the body and because of that any adverse effect of a substance or disease and malnutrition conditions can be indicated by a shift in the normal blood chemistry range of a healthy organism.

The innate defence mechanism of fish is usually negatively affected during stressful situations, and this may predispose a fish to profound metabolic disturbances as well as result in abnormal functioning of the neuro-endocrine and related systems. Analysis of the blood profile is an important diagnostic tool that can provide information about the health status of fish (Blaxhall & Daisley, 1973; Benerjec & Kumari, 1988; Rehulka, 2000).

22

As far back as 1958, Weinreb (1958) used changes in the blood profile of rainbow trout, *Salmo gairdneri* (R) as a means of assessing the systemic response of the fish to experimental conditions. Hrubec *et al*. (2000) reported haematological and blood chemistry reference interval for cultured tilapia (*Oreochromis* hybrid).

Stress in fish just like in other higher animals, leads to changes in the homeostasis of the organism. These changes are manifested in the form of variations in haematological parameters. Indices such as red and white blood cell counts, haematocrit and haemoglobin concentrations are all subject to variation in stressful conditions such as parasites and diseases (El-Feki *et al.*, 2000; Movahed *et al.*, 2012), environmental conditions such as temperature changes, dissolved oxygen (Roche & Bogé, 1996; Affonso *et al.*, 2002; Silkin & Silkina, 2005), and contaminants (Masson *et al.*, 2002; Parma *et al.*, 2007). Salinity also has an impact on the haematology of fishes (Akinrotimi *et al.*, 2012). Changes in blood parameters are an indicator of variation in biochemical processes induced by xenobiotics in animals including fish. According to Ayotunde *et al*. (2011), changes in the blood parameters and the histological architecture of tissues are employed to assess the disruption of homeostasis in fish due to toxicity. Pathology associated with blood values includes macrocytosis which is increase in mean corpuscular haemoglobin while microcytosis which is its decrease is associated with iron deficiency. Total protein, albumin, bilirubin, calcium, and phosphate analyses are used to test for liver function and calcium metabolism. It is believed that changes of blood constituents could adversely affect the performance of the fish. Likewise, changes in plasma levels of steroid hormones most often leads to corresponding changes in the immune status and health of the fish (Harris & Bird, 2000).

2.3: External factors affecting reproduction in tilapia

The environmental condition of fish plays an important role in its growth and reproductive performance. Reproductive processes such as gonadal maturation and spawning behaviour are known to start in response to environmental stimuli such as temperature, photoperiod, and the amount of rainfall among other external factors.

a)   Water temperature and dissolved oxygen

Water temperature plays a positive role in initiating gonadal maturation, egg and fry development in tilapia fish. According to Rana (1990), increasing temperatures accelerates while decreasing temperatures retard egg/fry development. The positive influence of temperature on reproduction is manifested in the seasonal pattern of spawning exhibited by *O. mossambicus* in that reproduction of the fish can be initiated by increasing the water temperature (Cornish, 1998). Research has shown that oocytes development and gonado-somatic index are both significantly influenced by elevated temperature regimes. Poor environmental conditions such as unfavourable

water temperature variations (Wang & Tsai, 2000) and low dissolved oxygen levels are known to cause regression of the gonads and interruption of reproductive cycles in fish (Madu, 1989). The optimum temperature for optimal growth and feed conversion ratio (FCR) for tilapia is between 26 – 30°C (Azaza *et al*., 2008), with certain members of the family like *O. aureus* that can tolerate very low temperature regimes (Pruginin *et al.*, 1975).

b)   Photoperiod

Photoperiod is one of the major environmental determinants of reproduction in fish. The pineal gland through its secretory product, melatonin influences the light-dark rhythm in most vertebrates including fish. Gonadal activities are known to be influenced by photoperiod and light intensity. According to Campos-Mendoza *et al*. (2004), photoperiod exerts an influence on reproduction in fish by affecting the brain-pituitary-gonadal axis.

However, there are conflicting reports on the nature of the influence exerted by photoperiod on reproductive parameters. Campos-Mendoza *et al*. (2004) reported production of significantly larger eggs in Nile tilapia (*O. niloticus*) subjected to normal day lengths, when compared to fish exposed to short day lengths, long day lengths or continuous illumination. They also found that total fecundity and relative fecundity were significantly higher in those reared under long day lengths. On the contrary, Rad *et al.* (2006) reported that long-day photoperiods significantly increases the growth rate of tilapia especially at fingerling stage but significantly decreases reproductive parameters such as gonado-somatic index and oocytes size. The implication of this according to them is that continuous light regimes may favour somatic growth while suppressing gonadal development. Manipulations of the photoperiod and light intensity have been successfully applied in controlling reproduction in Nile tilapia (*O. niloticus*) (Campos-Mendoza *et al.*, 2004; Biswas *et al.*, 2005).

c)   Rainfall

In the tropics most fish spawn during the rainy season and this may be due to increased water levels. Studies have shown that increased water levels have a positive influence on the frequency of spawning and general reproductive output (Bhujel, 2000).

d)   pH

Bicarbonate and carbonate anions present in the water determines its pH buffer system (De Holanda Cavalcante *et al*., 2010). The suitable pH value to tilapia culture for optimum growth and production range between pH 7 – 8 (El-Sherif & El-Feky, 2009) while the lower and upper lethal levels of pH for tilapia are 4 and 11 (Bhujel, 2000). There are few literature on the influence of pH on the reproductive processes in fish, however (Romer & Beisenherz, 1996) reported higher proportion of male among Apistogamma species at an acid pH (4.5).

24

e)   Stocking density

There is inverse relationship between stocking density and reproductive performance in fish. In tilapia culture a stocking density of 2 to 6 fish/ m^2 is the ideal for optimum performance (Bhujel, 2000) and exceeding this, will lead to a progressive decline in spawning.

f)   Diet

Adequate nutrition in quality and quantity is essential for optimum reproductive performance in fish just like in livestock production. A prolonged period of inadequate protein in the diet of brood fish will invariably lead to decline in the production output. The optimum level of protein for tilapia brood stock is 32% (Gunasekera *et al*., 1995). Also the effect of dietary vitamin supplementation on the reproductive performance of tilapia has been extensively reported (Mohamed *et al*., 2003; Alkobaby, 2008). Equally the quality and quantity of eggs spawned by brood fish are greatly improved with phosphorous and calcium in the diet (Mohamed, 2013). Lipids have also been shown to positively influence the reproductive performance of fish (Izquierdo *et al*., 2001). The lipids are also important in brood stock management and its deficiencies can inhibit oocytes production and survivability (Suloma & Ogata, 2012).

However, despite the good culture potential of the tilapias as a food fish, precocious maturation and indiscriminate breeding in mixed sex populations result in overcrowding of ponds and stunted growth of the fish (Toguyeni *et al.*, 2002).

2.4: Methods to control indiscriminate spawning in tilapia farming

a)   Polyploidy

Polyploids refer to organisms with one or more numbers of additional chromosomes. Triploid which is a type of polyploidy has three sets of homologous chromosomes in an individual. Triploid fish are generally sterile (Piferrer *et al.*, 2009). Polyploidy in aquaculture are applied to mitigate the effect of precocious maturation and indiscriminate spawning especially in tilapia culture to control reproduction and thereby improve growth and production. The process induces functional sterility in that it suppresses the maturation of gonads in females while products of the fertilization using triploid males are not viable.

b)   Sex reversal in fish (monosex male or female population)

Sex reversal converts genotypic females into phenotypic males (Toguyeni, Fauconneau, *et al.*, 2002) or vice versa. In masculinization also known as all-male or mono-sex production, the genetic females are induced to develop as functional males but the genetic males are not affected and therefore develop normally as functional males (Pandian & Varadaraj, 1987). Also in all female monosex production (feminization), the genetic males are induced to develop as functional females but the genetic females are not affected and so develop normally as functional females. Hormonal sex reversal or inhibition of gonadal growth is achieved through application of steroid before sexual differentiation or during the period of gametogenesis. Sex differentiation in fish is the change from primordial germ cells (gonads) to either ovary or testes (Viñas *et al.*, 2013). According to Afonso *et al*. (2001), male and female sex differentiation is modulated by androgen and estrogen hormones, respectively. Sex reversal can be achieved by the application of steroid hormones during the sexually undifferentiated stage (Rothbard *et al*., 1987). Androgen hormone sex reversed fish has a higher weight at harvest than their untreated counterparts (Macintosh *et al*., 1985). Since sex reversal processes take place during the period of sex differentiation the sex reversed fish is permanent.

Sexual determination or differentiation is a process by which the bi-potential gonad transforms to form either the testes or ovary. In fish the process is under the strong influence of environmental factors such as temperature, population density and pH (Guerrero-Este´vez & Moreno-Mendoza, 2010). Unlike mammals that have a stable sexual determinism, fish are flexible to the extent that even genetically determined sex when subjected to environmental influences can develop to opposite sex phenotypically (Baroiller & D'Cotta, 2001). Temperature is the environmental factor that has the most pronounced influence on fish sex determination (Baroiller *et al.*, 2009). Sex reversal processes are undertaken in food fish production to favour the sex that has the greatest growth potential which will ultimately lead to greater profitability of the enterprise. For example, the male grows bigger in cichlids, while in the salmonids and cyprinids it is the females that exhibit greatest growth (Piferrer, 2001a). Male *O. mossambicus* grows faster than their female counterparts and therefore all-male culture is usually preferred when production of food fish is the primary concern (Macintosh *et al*., 1985). Many factors have been attributed with the differential growth capabilities between the male and female tilapia, these factors include diversion of greater percentage of metabolic energy to breeding processes in females and androgens in male having higher anabolic effects (Toguyeni, Fauconneau, *et al.*, 2002). The advantages of production of monosex tilapia include elimination of reproduction, increased growth rate and possible reduction of variation in harvest size (Beardmore *et al*., 2001). However, Kirankumar & Pandian (2002) were of the opinion that sex reversed fish exhibits remarkably slower growth than the normal males.

c)   Manual sexing

Production of all male or all female tilapia grow out have been undertaken by manually separating the sexes before sexual maturation and subsequent spawning can take place. The separation of the sexes is usually carried out by careful examination of the urogenital papillae of each fish. According to Pandian & Varadaraj (1987), one orifice is present in the external genitalia of male *O. mossambicus* while there are two in females. Sometimes dyes are used to highlight the differences in papilla structure of the external genitalia of the fish during sexing. The draw back in manual separation of sexes as a means of all male tilapia production is that though the technique may appear simple, the urogenital papillae of tilapia are very difficult to distinguish between male and female especially if the person doing the separation is not an expert. Even when an experienced person is doing the sexing, mistake can easily be made in the separation and this can render the whole exercise ineffective since even a few females in all male production system can lead to breeding and subsequent overpopulation thereby predisposing to differential sizes at the time of harvest. Also manual sorting of tilapia fingerlings to separate the sexes in order to grow male progeny only not only consumes time but wastes the females (Pruginin *et al*., 1975).

d)   Hybridization

The origin and use of hybridization to produce all male progeny came accidentally when tilapia breeders in Israel discovered that offspring from certain crosses of certain species tends to have a higher male ratio than females (Pruginin *et al*., 1975). Some of the hybrids from certain crosses yield variable percentage of male progenies and according to Fishelson (1962) as cited by Pruginin *et al*. (1975), it was the cross between *Oreochromis niloticus* female and *O. aureus* male that produced 100% all male progenies. However, the constraint in the use of hybridization to produce all male tilapia progeny is the inability to maintain a parent stock which will consistently produce 100% male offspring. This is because subsequent crosses using the male from the previous 100% male hybrid seldom produces offspring of more than 90% male (Pruginin *et al*., 1975).

e)   Genetic manipulation of gender (YY super male technology)

The technique of production of all male tilapia through the YY super male technology was developed through a collaboration of scientists from University of Wales, Swansea, United Kingdom and the Central Luzon state University, Philippines (Mair *et al.*, 1997). The technology involves the production of males with YY genotype which when crossed with the normal XX females will result in all the progeny being normal males with XY genotype. The procedure for the production of the YY super males include;

- Sex reversal of genetic males into phenotypic females using hormone (17β-estradiol) i.e females with XY genotype.

27

- The XY females then crossed with normal males (XY) to produce a mixture of XX, XY, and YY in the ratio of 1:2:1. The genotype of each progeny is determined by progeny testing.
- The YY progeny are then crossed with feminized males (XY female) to produce YY and XY in the ratio of 1:1 (50:50).
- Sex reversal of the YY genetic males to phenotypic females using hormone (17β- estradiol).
- When the YY females are crossed with YY males, the products are all YY males which when crossed with the normal females (XX) will yield all normal XY males which are then stocked for grow out.

Super male (YY male) technology seems a veritable alternative to hormonal sex reversal but then the technique and expertise needed for the YY technology.

f)   Temperature and pH

Sex differentiation can be modulated by environmental factors such as temperature (Viñas *et al.*, 2013) therefore temperature variations have also been used in sex reversal in fish. Elevation of culture temperature (heat shock) before and during sex differentiations will lead to masculinization (all male production) while reduced temperature (cold shock) predisposes to feminization (all female production). Elevated rearing temperature of $32 - 35^0$C before the completion of sex differentiation will skew the sex ratio in favour of male (D'Cotta *et al.*, 2001). In an experiment, Azaza *et al*. (2008) applied high temperature of 36.9^0C during the period of sex differentiation in tilapia and claimed production of $64.2 - 80\%$ male. *Oreochromis mossambicus* is one of the fish species in which high rearing temperature can induce sexual differentiation in favour of males and low temperature in favour of females (Wang & Tsai, 2000). Hydrogen ion concentration (pH) is another environmental factor that influences the sexual differentiation in fish though to a lesser scale compared to temperature. High pH (7.9) predispose to the population being skewed in favour of females while a lower pH (6.2) favour development of male population (Guerrero-Este´vez & Moreno-Mendoza, 2010).

g)   Administration of hormones

Sex steroid used for masculinization in fish include 17α-methyltestosterone, 19- norethynyltestosterone, 11-ketotestosterone (Yamazaki, 1983) and 17-methyldihydrotestosterone while feminization are carried out by use of 17β-estradiol, estrone, and di-ethylstilbesterole. However, according to Pandian & Varadaraj (1987), 17α-methyl testosterone is the androgen hormone mostly used for the induction of sex reversal in fish while 17β-estradiol is the estrogen of choice. The potencies of these various steroids vary from each other. Method of induction of sex reversal using hormone include immersion of embryonic stage (sexually undifferentiated) fry in water containing steroid, mixing the steroid in the diet of the undifferentiated fry (Devlin & Nagahama, 2002) or

by alcohol evaporation method (Pandian & Varadaraj, 1987). The dosage of sex reversing hormones such as methyltestosterone or methyldihydrotestosterone required to induce the desired level of sex reversal in fish is very vital and should be carefully determined since under dosage may not be effective while over dosage may predispose to feminization instead of the expected masculinization (Liu *et al.*, 2000) and this is due to the conversion of the circulating excess androgen to estrogen by cytochrome P450 aromatase enzyme (Ankley *et al.*, 2002). In a sex reversal experiment with Betta splendens, Kirankumar & Pandian (2002) reported that a dose of 900μg/l of 17α-methyltestosterone produced nearly 100% masculinization whereas a low dose of 100μg/l failed to reverse the sex of the fish. They concluded that the percentage of males increases with increasing dose of the hormone. However, different authors gave different effective dosage of the hormone; Yamazaki (1983) recommended 30mg/kg diet in *Oreochromis niloticus* and *O. mossambicus* and 30 – 60mg/kg diet for *O. aureus*.

There are conflicting opinions on the exact time of sex differentiation in *O. mossambicus*, while Pandian & Varadaraj (1987) opined that in *O. mossambicus*, irreversible sex differentiation takes place between 10 – 20 days post hatch and it is during this period that manipulation of the sex ratio can take place. Macintosh *et al.* (1985) wrote that gonadal differentiation in *O. mossambicus* occurs between 35 and 48 days post hatch therefore treatment should be such that will extend beyond the 48 days in order to obtain 100% sex reversal. However, Hiott & Phelps (1993) were of the opinion that initial size of the fry is more critical factor in the overall success of sex reversal using androgenic steroids. They obtained 95.7% male at the initial size of less than 11mm and 52.6% in the initial size of greater than 16mm in the same age group. One of the problems of monosex tilapia production using hormone is the inconsistency in the sex ratio obtained from such technique, for instance in an experiment Mubarik *et al.* (2011) included 50mg of 17α-methyl testosterone (17α-MT) per 1kg of diet and obtained 76% masculinization, Vera Cruz & Mair (1994) reported 95.4 – 98.4% when they added 60mg 17α –MT per kg diet while Ampofo-Yeboah (2013) applying the same hormone and dosage obtained 71.5%. In fact, earlier researchers on sex reversal using 17α-MT reported higher percentages of masculinization than the later workers. Tayamen & Shelton (1978) reported that they got consistently 100% all-male brood when they included 30 – 60mg of 17α-MT in 1kg of diet, also Yamazaki (1983) applying a dose of 20 – 30mg/kg reported 100% masculinization. On feminization, Rosenstein & Hulata (1993) using synthetic estrogen, di-ethyl stilbesterole reported that at any dose greater than 10mg/kg they got 100% female stock.

Despite the effectiveness of the synthetic hormone in sex reversal and subsequent increase in overall fish production, concerns have been expressed concerning the health implication of the use of hormone in food fish production. Apart from the suspected health hazards of employing synthetic hormones in food fish production, concerns have also been expressed on the fact that they are expensive and difficult to obtain (Jegede, 2010a).

Because of these reasons efforts are being made to substitute synthetic hormones with less harmful and environmental friendly plants sources to manipulate the gender of fish. Pawpaw (*Carica papaya*) seed meal is one of the plants phytochemical that have attracted research in this regard (Ampofo-Yeboah, 2013).

2.5 Endocrine disrupting substances

An endocrine disrupting substance is a chemical substance originating outside an organism that exhibit an adverse effect on the production, distribution or function of hormones. Many anthropogenic chemicals have the capacity to disrupt endocrine systems and they do that by mimicking the action of steroid hormones thereby affecting the hormonal profile of fish (Schwaiger *et al.*, 2002). They affect systems that are normally regulated by estrogen, androgen and thyroid hormones. Since the steroid hormones are involved in the regulation of the GnRH – FSH/LH system through positive and negative feedback mechanism (Zohar *et al.*, 2010), these anthropogenic chemicals equally affects the reproductive capacity of an organism by interfering in the neuroendocrine system. The effects of endocrine disrupting chemicals include reduced viability of eggs, high mortality rate of fry /eggs and reduced hatching rate (Schwaiger *et al.*, 2002). Glyphosate based herbicides are widely used to control weeds around compounds, in farms and even around fish ponds (Koakoski *et al.*, 2013). These herbicides or their degraded by-products are regularly washed into fish ponds or other water bodies by flash flood. Exposure of fish to sub lethal concentration of these herbicides is known to disrupt the endocrine physiology of the fish thereby leading to disruption of normal homeostasis including that of reproduction. Sexually undifferentiated gonads are more prone to disruption by endocrine active chemicals (Van Aerle *et al.*, 2002). Glyphosate compound disrupts endocrine system of fish by blocking the cortisol response thereby reducing their capacity to withstand stress and maintain homeostasis (Koakoski *et al.*, 2013). Endocrine disruption in fish is indicated by the presence of significant level of vitellogenin in males or elevation of its levels in females. Unlike the female fish where vitellogenin is always present and easily detected in the blood especially in sexually matured fish, its presence in the male is usually very insignificant. Vitellogenin synthesized in the liver is incorporated to the growing oocytes where it serves as egg yolk precursor. The level of the vitellogenin in the serum of fish is measured using enzyme linked immuno-sorbent assay (Van Aerle *et al.*, 2002). Additionally, a significant increase in the levels of estradiol in males and testosterone in females demonstrates the exposure of fish to anthropogenic chemicals (Schwaiger *et al.*, 2002).

2.5.1 Plants as endocrine disrupting substances

Phytoestrogens are natural products of some cultured and wild plants that have the ability to modulate the endocrine system by exhibiting estrogenic and/or androgenic activity (Rearick *et al.*, 2014). Because they modulate the endocrine system, phytoestrogens are sometimes referred to as endocrine disrupting compounds

(EDC). The mode of action of EDC includes interfering with the enzymes responsible for biosynthesis of steroid hormones (Ribeiro *et al.*, 2012). Phytoestrogens are classified into isoflavones, coumestans and lignans (Clotfelter & Rodriguez, 2006). Many plants contain chemical substances that have pharmacological properties some of which are beneficial while others have adverse effects. Studies have shown that some of these phytochemicals can be used as reproductive inhibitory agents. Garg *et al*. (1998) used neem (*Azadirachta indica*) seed oil as an intrauterine contraceptive in female wister rats while Gbotolorun *et al.* (2008) reported antifertility effects of extracts from the flower of the same plant on female Sprague – Dawley rats. Other workers (Parshad *et al.*, 1997; Aladakatti & Ahamed, 2005) reported that intraperitoneal injection of steroidal extract of neem leaves resulted in impaired spermatogenesis, increased abnormal spermatozoa and decreased motility in male wistar rats. In an experiment, Jegede & Fagbenro (2008) studied the effect of inclusion of 0, 0.5, 1, 1.5 and 2g of neem leaf meal per kg of basal diets, respectively, for 60 days and reported a progressive decrease in reproductive traits with increasing dietary content of neem leaves. They concluded that the inclusion leads to destruction of testes and ovarian tissues. Even in humans, Khillare & Shrivastav (2003) reported spermicidal activity of neem. The action of neem are not only deleterious, beneficial effects have also been reported. Raji *et al.* (2004) reported antiulcer activity of neem extract in rats. Other plants with reported antifertility properties include pregnancy terminating effects of *Jatropha curcas* in rats (Goonasekera *et al.*, 1995), use of aqueous extract of *Moringa oleifera* to effectively prevent implantation in rats (Shukla *et al*., 1988). One of the remarkable claims in this connection was by Nath *et al.* (1992) who reported 100% abortificient activity of *Moringa oleifera* extract on rats. Writing on the effect of *Lagenaria breviflora* on the spermatogenesis of Wistar rats, Saba *et al.* (2010) found that the extract from the plant adversely affects andrological parameters of the rat such as morphology and motility of the spermatozoa and sperm count.

There are also reports on the activities of these phytochemicals from various plants on fertility and wellbeing of fish. Dongmeza *et al.* (2006) reported reduced growth performance in Nile tilapia fed diet containing *Moringa oleifera* while Makkar *et al*. (2007) used diet containing 300p.p.m. of quillaja saponin to suppress reproductive activities in Nile tilapia.

2.5.2 Pawpaw (*Carica papaya)* as a source of phytochemicals

Carica papaya Linn (Caricaceae family) popularly called pawpaw, is widely available in sub-Saharan Africa and other tropical and subtropical areas worldwide. The popularity of the plant is due to its fruit, which when ripe are yellow and succulent and very nutritious. Mature unripe pulp of pawpaw contains the phytochemicals saponins, alkaloids, terpenoids, flavonoids, glycosides, steroids and cardenolides which are responsible for its medicinal properties (Oloyede, 2005; Ezike *et al.*, 2009). Likewise, the chemical composition of the seed of

pawpaw consists of fatty acids, crude proteins, crude fiber, papaya oil, carpaine, benzyl isothiocyanate, benzyglucosinate, glucotropacolin, benzylthiourea, hentriacontane, β-sitosterol, caricin and enzyme myrosin (Krishna *et al*., 2008). Apart from the nutritive aspect, different parts of the *C. papaya* tree and even the seeds have been known to possess a wide range of pharmacological properties especially antibacterial, antifungal, pesticidal and anti-fertility (Eno *et al.*, 2000; Lohiya *et al.*, 2005; Doughari *et al*, 2007). Doughari *et al*. (2007) reported antibacterial activity of bioactive compounds of the root extract of pawpaw against some pathogenic bacteria, they postulated that the extract may be used to treat gastroenteritis, urethritis, otitis media, and typhoid fever and wound infections. Emeruwa (1982) agreed that fruit and seed extracts of pawpaw has antibacterial properties. It is also claimed that the fruit juice of pawpaw has a depressant effect on blood pressure (Eno *et al.*, 2000).

Pawpaw seeds has also been credited with molluscicidal activities against freshwater snail *Lymnaea acuminata* (Jaiswal & Singh, 2008). Farias *et al.* (2007) reported that the crude extract of seeds of pawpaw exhibited significant inhibitory activity against cowpea weevil (*Callosobruchus maculatus*). Ayotunde & Ofem (2008) opined that pawpaw seeds are poisonous to fish, and have been used to control overpopulation in tilapia culture systems.

In a study by Panzarini *et al.* (2014), whole extract of pawpaw seeds were compared to subfractions of the seed water extract to determine whether the respective treatments differed in terms of the antioxidant activity to protect Detroit 550 fibroblasts against oxidative stress. They concluded that the subfractions of the seed water extract was equally effective in their antioxidant activity in protecting the fibroblasts against the damage caused by free radicals.

2.5.3 Antifertility effect of Pawpaw (*C. papaya*) seed on laboratory animals

The antifertility effect of pawpaw (*C. papaya*) seeds have been extensively studied in albino rats (Joshi & Chinoy, 1996; Udoh & Kehinde, 1999; Adebiyi *et al.*, 2003; Manivannan *et al.*, 2004; Verma *et al.*, 2006), however there are conflicting reports on the efficacy or otherwise of the phytochemical in inducing infertility and/or toxicity on the laboratory animal. Adebiyi *et al*. (2003) reported degeneration of the endometrium and myometrium in the uterus of rats treated with pawpaw seed extract, thereby predisposing them to infertility. Manivannan *et al.* (2004) included 10mg per day of chloroform extract of pawpaw seed in the diet of albino rats for 150 days and recorded decreased motility and sperm count. The pathological effect of the pawpaw seed have also been reported on the pituitary glands. Udoh *et al.* (2005) observed marked hypertrophy of the FSH and LH cells of the pituitary glands. However, Chinoy *et al.* (1994) stated that the aqueous extract of pawpaw seed given 5mg/kg

body weight per day intramuscularly and 20mg/kg body weight per day orally to male albino mice for 60 days did not have a toxic effect, and did not affect the reproductive organs and kidneys. They were also of the opinion that the levels of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), protein and cholesterol were within the normal range suggesting that the extract has no effect on liver function or cholesterol and protein metabolism. They concluded that even though the aqueous extract is a potent male contraceptive agent, it has no harmful side effect. Lakshman & Changamma (2013) reported that pawpaw seed extract significantly decreased the cholesterol level in the testes leading to decreased steroidogenesis and ultimately decreased spermatogenesis in albino rats fed high levels of the extracts. They concluded that pawpaw seed contains oleanolic glycoside which causes sterility in male rats by inhibiting the steroidogenesis which leads to anti-spermatogenesis. Jaiswal & Singh (2008) were of the opinion that the efficacy and /or toxicity of the pawpaw seed depends on the dose and duration of the application. Agreeing on the dose dependent toxicity of pawpaw seed, Udoh & Kehinde (1999) reported that at a high dose of 100mg/kg body weight for 8 weeks, pawpaw seed induces degenerative lesions among other pathological conditions in the gonads of male albino rats while at a low dose milder effects occurred. However, Mansurah *et al.* (2009) reported that fermented seeds of pawpaw does not adversely affect the reproductive activities in Wistar rats. Joshi & Chinoy (1996) stated that the benzene extract of pawpaw seeds, although it had a reversible antifertility effect, did not affect ovarian steroidogenesis in female rats.

In a study on albino rats, Lohiya *et al.* (2005) investigated whether the ethyl acetate subfraction and methanol subfraction prepared from pawpaw seeds, differed in their ability to elicit an anti-fertility effect in albino rats. Their results indicated that the two subfractions were equally effective in disrupting spermatogenesis, and neither of the treatments had any toxic effect.

2.5.4 Antifertility effect of Pawpaw (*C. papaya*) on fish species

The effect of phytoestrogen exposure in fish includes impairment of the reproductive system in adults and outright sex reversal in larvae (Ampofo-Yeboah, 2013). The impairment of the reproductive activities manifests as infertility, reduced fecundity, ovo-testes in females and vitellogenin induction in males (Ribeiro *et al.*, 2012). Estrogenic phytoestrogens are capable of exhibiting anti-estrogenic tendencies in the presence of endogenous estrogens, and that may explain the sex reversal action of some phytoestrogens like those contained in pawpaw seed powder (Clotfelter & Rodriguez, 2006). Compared to the literature on the anti-fertility effect of pawpaw seed on laboratory animals, there are relatively little information on the effect of pawpaw seed powder as part of fish diets on the reproductive potential of fish. However, Hossam & Wafaa (2011) used 6g/kg/day to induce permanent sterility, and 3g/kg/day to induce reversible sterility in Nile tilapia (*O. niloticus*).

It has been found that pawpaw seed meal fed to sexually undifferentiated fry of Mozambique tilapia (*O. mossambicus*) was able to skew the sex ratio in favour of males (Ampofo-Yeboah, 2013), however this experiment was confined to only one inclusion level (15g/kg of basal diet) of pawpaw seed meal, and therefore the optimum level could not be determined. There is also shortage of information on how the phytochemicals contained in the pawpaw seeds affects liver function, the steroid hormone profile and blood haematological and biochemical profiles of Mozambique tilapia. The study therefore investigated the potential of different inclusion levels of pawpaw seed meal to achieve the optimal level of masculinization in Mozambique tilapia, and to assess whether the established maximum inclusion level will affect the normal functioning of the liver and hypothalamus-pituitary-gonad (HPG) axis, as indicated by abnormal haematological and blood biochemistry profiles. Information generated will contribute significantly to the formulation of standard culture protocols that will potentially enable especially rural poor farmers to culture Mozambique tilapia as cost-efficiently as possible.

2.6 Conclusions

Fish and fisheries products constitute an affordable source of animal protein worldwide. Tilapia popularly referred to as "aquatic chicken" has the potential to contribute substantially to solving the problems of the deficit in protein intake, especially in Sub-Saharan Africa that is in dire need of food security. Tilapias are easily propagated and through its cultivation, can contribute to poverty alleviation among the rural poor communities. However, the precocious maturation inherent in the species predisposes to indiscriminate spawning leading to overpopulation of pond systems and consequent stunted growth, which ultimately result in a decreased production efficiency. Efforts to mitigate this shortcoming through all male mono-sex culture using hormonal sex reversal techniques are largely not accessible to the rural subsistence fish farmers. Pawpaw (*Carica papaya*) seed contains some phytochemicals that are capable of disrupting the endocrine systems of animals including fish thereby leading to reproductive impairment and possibly sex reversal in sexually undifferentiated fish. The masculinization and reproductive inhibition properties of pawpaw seed meal which abound worldwide and easily accessible to the rural poor communities are discussed including its possible effect on health and wellbeing of the fish.

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Chapter 3

General materials and methods

The study consisted of 3 experiments: the first part was to determine the optimal inclusion level of pawpaw (*Carica papaya*) seed powder in the diet of Mozambique tilapia (*Oreochromis mossambicus*) to produce all-male populations to ensure a uniform population size at harvest. The second and third part of the study assessed the effect of pawpaw seed powder on the function and integrity of the reproductive system, and liver function and integrity of Mozambique tilapia males and females, respectively.

This chapter aims to provide a comprehensive description of the methodologies used in the respective experiments, and in the respective chapters, a brief description will again be given, so a certain degree of repetition will occur.

3.1 Experimental location

The study was undertaken at the Aquaculture Research Facilities situated on the Welgevallen Experimental Farm of Stellenbosch University, Stellenbosch, South Africa. The GPS coordinates of the facilities are 33°56' 33.95" S and 18°51'56.15 "E. The climate is considered as being Mediterranean, with minimum temperature (6^0C) in July and maximum (40^0C) in January, while annual rainfall is 673mm (Source: World Climate Online).

3.2 Experimental systems and maintenance of culture conditions

The first experiment was carried out in a two tier water recirculation system (Plate 3.1). The recirculatory aquaria system (RAS) consisted of 72 glass aquaria. Each aquarium is 57x53x40cm (LxWxH) in size, and has a capacity of 120.8L. Aeration, filtration and water heating facilities are incorporated into the design. Uneaten food and solid wastes were removed from the culture water using a mechanical filtration system, while the bio-filtration system removes ammonia and nitrites. These filtration systems ensures no cross contaminations of phytochemicals from uneaten food and/or excreta from one culture tank to another. The culture tanks were aerated using a 1.1kW blower (FPZ Effepizeta, SRL, Model SCL V4, Incorezzo, Milano, Italy), and a 1000kW heating element positioned in the sump, was used to maintain the temperature of the culture water.

Plate 3.1   The two tier glass water recirculation aquarium system (RAS) used to conduct Experiment 1.

The second and third experiments were conducted in a plastic water recirculatory system built in a glass house, as indicated in Plate 3.2. This system consisted of 88 plastic tanks, with the dimensions of each tank being 70x40x38cm (LXWXH), and a capacity of 90 litres. Similar to the glass RAS system, aeration, filtration and water heating facilities are incorporated into its design. Uneaten food and solid wastes were removed from the culture water using the mechanical filtration system, while the bio-filtration system removes ammonia and nitrites. The culture tanks were aerated using a 1.1kW blower (FPZ Effepizeta, SRL, Model SCL V4, Incorezzo, Milano, Italy), and a 1000kW heating element positioned in the sump, was used to maintain the temperature of the culture water. The water recirculation system provides constant water flow rate of 13.6±1.9L/second while the aerating system supplies a constant airflow of 7.74mg/L/second to each aquaria. The physicochemical parameters (water temperature, dissolved oxygen, pH and conductivity) of the culture water were monitored daily. The monitoring of the physic-chemical parameters especially temperature, DO and pH in this study was premixed on the fact that they are the environmental factors most relevant to sex differentiation in fish. This was elaborated more in the literature review section.

Plate 3.2 The plastic water recirculation aquarium system (RAS) used for Experiments 2 and 3.

A digital YSI ProODO instrument with a fitted probe was used to monitor dissolved oxygen levels. An YSI Ecosense conductivity meter (Model: EC300, YSI Inc., Yellow Springs, USA) was used to monitor the water temperature and conductivity. A Crison ICR12502 pH meter fitted with an ICR15053 electrode (HACH) was used to monitor the pH of the culture water. The recorded water quality parameters, presented in Table 3.1 and Table 3.2 respectively, were within the tolerable limits for tilapia culture (Timmons & Losordo, 1994).

Table 3.1. Water quality parameters maintained during Experiment 1 in the glass recirculation aquarium system.

Parameter	Mean ± SD	Range
Dissolved oxygen	4.98 ± 0.73	3.2 – 6.10
Temperature	24.48 ± 1.46	22.2 – 26.70
pH	6.42 ± 0.18	6.12 – 6.72
Conductivity	210 ± 8.02	184.3 – 228.50

50

Table 3.2. Water quality parameters maintained during Experiments 2 and 3 in the plastic recirculation aquarium system.

Parameter	Mean ± SD	Range
Dissolved oxygen (mg/L)	6.77 ± 0.78	4.80 – 7.80
Temperature (^{0}C)	26.03 ± 0.61	24.30 – 26.70
pH	5.73 ± 0.28	4.80 – 6.53
Conductivity	236.78 ± 13.51	210.30 – 260.80

3.3 Experimental diets

3.3.1 Preparation of pawpaw seed powder

Ripe pawpaw fruits (Plate 3.3) were purchased from fruit vendors in Stellenbosch Western Cape, South Africa. Because the fruits were purchased from fruit vendors, the actual variety of the pawpaw cannot be determined with certainty. The fresh seeds were removed from the fruit, and after rinsing in distilled water to remove the attached membranes of the fruit, the black seeds were spread open on black refuse bags, and left indoors to dry. The dried seeds were then ground to obtain a powder using a laboratory grinder (Knifeter 1095, FOSS). The ground seeds were subsequently passed through a laboratory sieve (SABS ISO3310, Model Minor, serial number: 371293, aperture: 500µm when preparing the fry diet, and serial number 5851325, aperture 1.0mm when preparing the juvenile feed) to obtain a fine powder. The pawpaw seed powder was packaged in appropriate containers (zip lock bags), labelled appropriately, and stored in a cool dry place until later use.

Plate 3.3 Examples of the fresh pawpaw fruits acquired for the preparation of the pawpaw seed meal used in the respective experiments.

51

Composition of treatment diets

The basal (control) diet (BD) consisted of a commercially available tilapia diet (Aqua-Nutro, Nutroscience (Pty) Ltd, Malmesbury, South Africa) with 40% crude protein. The nutritional composition of the BD is presented in Table 3.3.

Table 3.3. Ingredient composition of the basal diet according to the production company.

Ingredient	Composition (g/kg)
Protein	400
Lipid	80
Moisture	120
Fibre	40
Calcium	30
Phosphorus	7

The experimental diets were mixed according to the different inclusion levels evaluated in the study, and all experimental diets were thoroughly blended in a Macadams mixer (model: SM-401). To enable pelleting of the feed, 200mL lukewarm water was added per kg of the feed during the mixing stage. The PSP-BD mixture was pelleted using an extruder (Trespade; model: 22 profess № 04/00090), and oven-dried in a CFW Envirowatch 5 oven (model: Ø560) for 12 hours at 60 ^{0}C. The final pelleted diets were then packaged in zip lock bags, and stored in an airtight container under cool dry conditions until later use. The quantity of feed prepared each time was done as such to allow for a maximum shelf life of two weeks.

Preparation of the 17 α-methyltestosterone

The stock solution was prepared by dissolving 60 mg of 17 α-methyltestosterone in 600mL of 95% alcohol which was then sprayed on 1 kg of basal diet and mixed thoroughly. The mixture was spread on a black refuse bag and allowed to dry indoors.

3.4 Experimental animals and husbandry

Experiment 1

Yolk sac fry of the red strain Mossambique tilapia (*Oreochromis mossambicus*) were used for the study. This strain together with grey-coloured strain constitutes the most commonly farmed tilapia species in Southern Africa. The preference of *O. mossambicus* to farm with was due to its favourable growth compared to other

indigenous species in Southern Africa (Brink *et al.*, 2002). The strain was chosen for the study because of their easy availability and being indigenous to Southern Africa requires no import permit to obtain. The fish were obtained from the Rivendell Hatchery, Grahamstown, South Africa, and were about one to two weeks old. Upon arrival at the Welgevallen Experimental Farm of the University of Stellenbosch, the fry were acclimatized for one day before assignment to the respective treatment groups. The stocking density for this part of the overall study was 60 swim-up fry of 1 to 2 weeks old per replicate (approximately 2 fish/m^2). Before stocking, 5 fry were randomly taken from each replicate and their weight and total length recorded. To avoid stocking a single progeny group in one treatment, fry were sorted and randomly divided into groups of 60 each, and then transferred to the treatment aquaria.

Feeding with the experimental diets started from 9th day post hatch when the hatchlings must have absolved their yolk sac. The fish were fed *ad libitum* three times a day (9.00 – 9.30h, 13.00 – 13.30h and 17.00 – 17.30h) with the experimental diets. The waste and uneaten food in the aquaria were carefully removed daily by siphoning and the tanks refilled with fresh water. The siphoning of uneaten food and cleaning of aquaria were done before the commencement of feeding each day. The experiment lasted from the beginning of the first feeding of hatchlings till 4 months (120 days) when the fish might have reached sexual maturity. According to Popma & Lovshin (1995), *Oreochromis mossambicus* becomes sexually mature as early as 3 months of age and at 15 g live weight. The groups that were fed with the experimental diet for 30 day were maintained with the basal diet from the 30th day (mean weight at this stage was 4.5±0.18 g) till end of the experimental period (120 days). Mean weight at the end of the experimental period was 37.22±1.63g.

Experiment 2 and 3

A total of 1 000 two month old *O. mossambicus* in the pre-vitellogenic phase (mean weight 24.81±8.54 g, mean total and standard lengths of 11.06±1.3 cm and 8.84±1.14 cm, respectively) were used for the study of the effect of *C. papaya* on the reproductive parameters and hormonal profile of the fish. The fish were obtained from the Rivendell Hatchery, Grahamstown South Africa.

The fish were stocked in a holding facility (rectangular fiberglass tanks of about 1000 L capacities) upon arrival at the Welgvallen Experimental Farm, where they were acclimatized for three weeks before being assigned to the respective treatment groups. The stocking densities were 50 fish (approximately 1.8 fish/m^2) per replicate and there were 4 replicates per treatment.

During the acclimatization period they were fed *ad-libitum* twice daily with standard (basal) tilapia diet. The fish were fed *ad libitum* three times a day (9.00 - 9.30, 13.00 – 13.30 and 17.00 – 17.30 hours) with a commercial tilapia feed containing different concentrations of the seed powder of *C. papaya*. The waste and uneaten food in the aquaria were carefully removed daily by siphoning and the tanks refilled with fresh water. On the day of stocking the entire fish in the whole treatments and replicates were weighed individually and their total and standard lengths measured using weighing balance and measuring board respectively.

3.5 Experiment design

Experiment 1

There were 12 experimental treatment groups based on the inclusion level of PSM and three replicates for each treatment. The treatment groups and their designation were as shown in Table 3.4.

Table 3.4 Inclusion level of pawpaw seed meal (PSM) and designation of treatment groups.

Treatment	Inclusion level	Designation
Basal diet (BD; negative control) for 4 months	No inclusion of PSM	BDM4
BD + 60mg 17α-Methyl testosterone for 1 month	No inclusion of PSM	MTM1
BD + PSM for 1 month	10g PSM/ kg BD	P10M1
	15g PSM/ kg BD	P15M1
	20g PSM/ kg BD	P20M1
	25g PSM/ kg BD	P25M1
	30g PSM/ kg BD	P30M1
BD + PSM for 4 months	10g PSM/ kg BD	P10M4
	15g PSM/ kg BD	P15M4
	20g PSM/ kg BD	P20M4
	25g PSM/ kg BD	P25M4
	30g PSM/ kg BD	P30M4

Data recorded

Sampling frequency

Sampling and recording of the morphometric data of a representative sample of each replicate was carried out every two weeks, and the sample size was 10 fish per replicate. The final sampling and taking of the morphometric of a representative sample in each replicate was undertaken at the 120th day and the mortality in each treatment was recoded so as to calculate the survival rate. Condition factor, growth and mortality rates were determined by using the following formulas below.

The condition factor (K) of each fish was calculated using the formula:

$$K = \frac{100W}{L^3} \quad \text{(Ricker, 1975)}$$

Where L = standard length (cm) and W = Body weight (g)

Specific growth rate was calculated using the formula:

$$SGR = \frac{LnW_f - LnW_i}{T \text{ (days)}} \times 100 \quad \text{(Tran-duy } et\ al.\text{, 2008)}$$

Where W_f = final weight and W_i = initial weight

Survival rate was calculated using the formula:

$$\text{Survival rate} = \frac{\text{Final number of fish}}{\text{Initial number of fish}} \times 100$$

Data from dissected organs

Gonad weight, Gonado-somatic index (GSI), egg diameter, fecundity, relative fecundity, liver weight and the Hepatosomatic index (HSI) of experiments 2 and 3 were determined according to the respective formulas listed below.

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

55

$$\text{Relative fecundity} = \frac{\text{Number of eggs spawned}}{\text{Body weight (g)}}$$

$$\text{HIS} = \frac{\text{Liver weight (g)}}{\text{Body weight (g)}} \times 100$$

Fecundity was estimated as shown below

Identification of sexes

Females and males from each replicate were identified and all fish were weighed at the time of sexing. Identification was done at the end of the experimental period when the fish might have grown to the size that will allow for visual sexing by external secondary sex characteristics. According to Pandian and Varadaraj (1987), one orifice is present in the external genitalia of male tilapia, while there are two in females. The sexing was done with the aid of a hand lens and each of the fish sampled was dissected to confirm the sex. The gonads were dissected out for histological preparation.

Data Collection

Length and weight measurement: The total and standard lengths of the specimen were measured using a measuring board graduated in centimeters. The total length was measured from the anterior most extremity of the fish to the end of the tail fin to the nearest centimeters. The standard length was measured from the most anterior extremity to the base of the tail fin. The base was identified by a crease when the tail was bent sharply from side to side. A top loading balance (Electronic Balance, UWE, HGS-300, capacity: 300 x 0.01g, Serial # P9440) was used to measure the body weight of the fish samples to the nearest gram. The mortality in different treatment groups were recorded, and also the number of females and males.

Experiment 2

The experimental set up based on the inclusion levels and duration of the feeding period of the experimental diet was as shown in Table 3.5.

Table 3.5 Inclusion level of pawpaw seed meal (PSM) and designation of treatment groups for experiment 2

Treatment	Inclusion level of PSM	Designation
Basal diet (BD; negative control) for 2 months	No inclusion	BDM2
BD + PSM for 1 month	10 g PSM/ kg BD	P10M1
	30 g PSM/ kg BD	P30M1
BD + PSM for 2 months	10 g PSM/ kg BD	P10M2
	30 g PSM/ kg BD	P30M2

Collection of blood samples

Blood samples were taken from four fish per replicate on the day of stocking to measure the level of the reproductive hormones. Two females and two males based on the appearance of their urogenital papilla were randomly selected from each replicate for blood collection and subsequent hormone analysis. Blood sampling and recording of the morphometric parameters were repeated on the day-30 post stocking and finally on the 60th day.

Blood samples were collected from the caudal circulation (Affonso *et al.*, 2002) with the aid of heparinized 3mL disposable plastic syringes and a 21 gauge disposable hypodermic needle. The protocol for the collection of blood using heparinized needle and syringe was as follows:

I.   Dilution of 0.5mL heparin in 500mL of saline;

II.   Syringe was filled with the heparin-saline solution, and left for a few minutes;

III.   Immediately prior to venepuncture, the syringe was emptied with enough little heparin remaining behind to prevent clotting.

The collected blood was transferred to 2mL (purple coloured cap) ethylene diamine tetra acetic acid (EDTA) vacutainer tubes. The plasma was collected by centrifugation at 3500 rpm for 10 minutes at 4 °C using an Eppendorf centrifuge (Model 5804R). The plasma was pipetted out, and transferred to Eppendorf tubes (200μL of plasma in each tube) and stored in the freezer at a temperature of -20°C until later analysis.

Quantification of the hormonal profile of *O. mossambicus*

The steroid hormone profile of each fish was quantified using ELISA kits. The reproductive hormones, 17β-estradiol and 11 keto-testosterone levels of each fish were quantified using ELISA kits specific for the quantitation of fish hormones. The 11-ketotestosterone hormone was assayed using fish specific 11-Ketotestosterone EIA kit (Item №: 582751; Batch: 0468795) manufactured by Cayman Chemical, USA while 17β-estradiol was assayed

using Fish Estradiol (E2) ELISA kit (Catalog №: CSB-E13017fh; Lot: C2489421809) manufactured by CUSABIO BIOTECH Co, China. The procedures for the assays were according to manufacturer's instructions, and were carried out in duplicate.

The summary of the assay procedure for the quantitation of 17β-estradiol includes

1. Reagent samples prepared as instructed
2. Set a blank well without any solution
3. 50μL standard or sample to each added to each well
4. 50μL horse radish peroxidase (HRP)-conjugate added to each well (not to blank well)
5. 50μL antibody added to each well
6. Incubated for 60 minutes at 37^0C
7. Aspirate and washed 3 times
8. 50μL substrate A and 50μL substrate B added to each well
9. Incubated for 15 minutes at 37 °C protected from light
10. 50μL stop solution added to each well and then read at 450nm, within 10 minutes.

Results of each ELISA assay were calculated using the professional soft "Curve Expert 1.3" to make the standard curve. The procedure for the quantitation of 11- ketotestosterone was also carried out according to the manufacturer's instructions.

For more details on the ELISA assays, please refer to the Appendix.

Measurement of reproductive parameters.

At the end of the 60 day experimental period, four fish from each replicate (two males and two females) were randomly selected and after recording their weights, standard and total lengths, the fish were dissected to determine the maturation of the gonads, fecundity, and egg diameter. The dissected gonads were measured and then preserved in 10% formalin solution prior to estimation of the fecundity and measurement of the egg's diameter. The diameter of 12 eggs randomly taken from the anterior, middle and posterior parts of the ovary, respectively, was measured using a binocular microscope. The long and short axis of each egg were measured and the mean taken as the diameter of the egg (Abdelhak *et al.* 2013).

Fecundity determination

Dissected ovaries were preserved in 10% formalin for 3 weeks, later they were gently agitated to separate the eggs from the ovarian tissues and then the formalin decanted out. The eggs were washed by adding clean water in a beaker containing the eggs, after gentle agitation, the water was filtered out. Entire eggs were put in a clean filter paper and weighed, a sub-sample of the eggs were weighed then counted. The fecundity of each female fish sampled was determined using the formula:

$$\text{Fecundity} = \frac{\text{Total weight of ovary}}{\text{Weight of sub sample}} \times \text{number of mature eggs in sub sample.}$$

<div align="right">(Gaikwad et al., 2009)</div>

Data Collection

Gonad weight, body weight, fecundity and egg diameter were recorded as explained above, and blood levels of sex hormones were determined as explained above. The haematological parameters and sex hormone assays were carried out as indicated below.

Experiment 3

Stocking and feeding of the juvenile experimental fish were as escribed for Experiment 2 above. There were also five experimental treatment groups with four replicates each. The treatment groups and the duration of the treatment were as shown in Table 3.6.

Table 3.6 Inclusion level of pawpaw seed meal (PSM) and designation of treatment groups.

Treatment	Inclusion level of PSM	Designation
Basal diet (BD; negative control) for 2 months	No inclusion	BDM2
BD + PSM for 1 month	10 g PSM/ kg BD	P10M1
	30 g PSM/ kg BD	P30M1
BD + PSM for 2 months	10 g PSM/ kg BD	P10M2
	30 g PSM/ kg BD	P30M2

The groups fed the PSM for 1 month was maintained on the basal diet from the 31st day to the end of the experimental period.

Measurement of morphometric parameters

The morphometric parameters were taken on the day of stocking, 30th and 60th days respectively. Blood samples were also collected from four fish (2 males and 2 females) from each replicate on the day 0, 30 and 60 respectively for the determination of the haematological parameters and blood chemistry assay. At the termination of the feeding period, two males and two females from each replicate were randomly selected, measured and their livers dissected out and after measurement, were preserved in 10% formalin for later histological processing and evaluation.

Determination of haematological parameters

Blood samples were collected from the caudal circulation (Affonso *et al.*, 2002) with the aid of heparinized 3mL disposable plastic syringes and a 21 gauge disposable hypodermic needle as shown in Plate 3.4. The collected blood was put into 4mL purple coloured EDTA vacutainer tubes. The haematological parameters analysed included the red blood cell (RBC) counts, haemoglobin (HGB), packed cell volume (haematocrit, HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), thrombocytes (platelets, PLT), white blood cell (WBC) counts, neutrophils (NEU), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS), basophils (BASO). The haematological parameters were all determined using an automated system, Abbot CELL-DYN system (model: 3700).

Plate 3.4 Blood sample collection from the caudal circulation for the determination of haematological profile of *O. mossambicus* fed PSM.

Blood Chemistry assay

Blood samples were collected from the caudal circulation with the aid of 3mL disposable plastic syringes and a 21 gauge disposable hypodermic needle. The collected blood was put into a non-heparinized serum separating vacutainer tube containing gel (yellow tubes) and allowed to clot. The serum was collected by centrifugation at 4000 rpm for 15 minutes at room temperature and then sent to the lab for analysis. The tests were done on a BECKMAN instrument (Model: AU5800) at the Pathcare Veterinary pathology laboratory. Cholesterol assay was based on an enzymatic colour reaction, glucose levels were determined by means of an enzymatic UV reaction, while albumin and total protein assays were determined by means of photometric methods.

3.6   Histological evaluation of gonads and liver

Collection of samples

Experiment 1

At the end the experimental periods, histology of the liver and gonads were prepared to determine the effects of the phytochemicals on their architectural integrity. Fish samples from each replicate (two males and two females) were selected, humanely sacrificed and the abdomen dissected. Tissue sections were dissected out from the ovary if the fish is female or testis if male then fixed in 10% buffered formalin with two changes at 24 hour intervals. For the histology of the liver, tissues were collected from two fish in each replicate (one male and one female).

Experiment 3

Fish samples from each replicate (two males and two females) were randomly selected, weighed, humanely sacrificed and the abdomen incised and the liver dissected out. After taking the weight the liver was fixed in 10% buffered formalin for the preparation of histological slides.

Processing of samples

The histology slide preparation was carried out at the laboratory of the Department of Physiology, University of Stellenbosch, South Africa. The histological method was as follows;

(1)   Dehydration – The fixed tissue samples were cut and embedded into cassettes and processed using an automated tissue processor (TISSUE TEK II) by dehydration in several changes of concentrated alcohol starting from 70%, 95% and then three changes of 100% alcohol at 1 hour intervals.

(2)   Clearing – Tissues were removed from alcohol and passed through three changes of xylene at 1 hour interval.

(3)   Filtration – Tissues were removed from xylene and kept in molten paraffin wax at the temperature of about 58^0 to 60^0C. Three changes of molten paraffin wax at 1 hour interval.

(4)   Embedment – Tissues were removed from molten paraffin wax and embedded in a fresh paraffin wax in an embedding instrument (LEICA EG 1160).

(5)   Blocking – Embedded tissues were cut to different cubes and then mounted in a prepared block.

(6)   Sectioning – Block embedded tissues were sectioned in a microtome (Reichert Jung, Heidelberg, Austria) at the thickness of 5μ and best sections were floated on warm water and then picked up with a pre-coated slide with glycerine albumen and then labelled appropriately.   This was followed by drying at room temperature for 24 hours.

(7)   Staining – sections were de-waxed in xylene, two changes at 3 minutes each and hydrated in 100% alcohol, 95% and 70% followed by immersion in water and stained with haematoxylin for 15 minutes. Stained slides were rinsed in water and differentiated in 1% acid alcohol followed by rinsing in water. Bluing of sections followed by leaving the sections in water for 5 to 10 minutes. Stained haematoxylin sections were finally counter stained in 1% eosin for 8 seconds and dehydrated rapidly in several changes of alcohol and followed by clearing in several changes of xylene rapidly. The slides remained in the last xylene until they were mounted using DPX. Each slide was finally examined under the microscope for histological evaluation of the tissue samples. A binocular microscope attached with NIKON digital camera (Model: DS-Fi1) was used for the evaluation.

Evaluation and interpretation of histological slides

Evaluation and interpretation of histological pathology of tissues are somewhat subjective but at the same time results are consistent if carefully carried out. For critical evaluation of histopathology of the gonads and liver associated with exposure to chemicals or phytochemicals, certain criteria have been developed by pathologists. The criteria are qualitatively described with severity of the pathological condition graded on a numerical scale. However, evaluation of the effect of phytochemical on the proportion of cells of an individual fish is inappropriate since variation among individual fish even in the same treatment is to be expected. Therefore the effects of phytochemical on gonadal or liver cell proportion are best assessed among the different treatment group as a whole.

Table 3.7 Grading criteria for the evaluation of the histopathological effect of pawpaw seed meal on the gonadal tissues of male *O. mossambicus* (adapted from USEPA, 2006; OECD 2009).

Grading	Condition	Description of characteristics assessed
0 - 1	Normal/ no effect	1. Normal spermatocytes in different stages of development 2. Intact interlobular interstitium and tunica albuginea 3. Intact testicular wall and collecting ducts.
1 - 2	Minimal effect	1. Increased or decreased number of spermatogonia relative to spermatocytes, spermatids and spermatozoa 2. Minimal Increase in interstitial cell 3. Onset of degeneration in testicular tissue
2 - 3	Moderate	1. Disintegrating sperm cells 2. Increasing necrosis of testicular cells 3. Collecting ducts devoid of spermatids
4 - 4	Severe effect	1. Presence of oocytes in the testis (testis-ova). 2. Degeneration of the testes characterized by cell shrinkage and vacuolated germ cells. 3. Hypertrophy of the interstitial cells 4. Disintegration of sperm cells 5. Granulomatous inflammation

Table 3.8 Grading criteria for the evaluation of the histopathological effect of pawpaw seed meal on the gonadal tissues of female *O. mossambicus* (adapted from USEPA, 2006; OECD 2009).

Grading	Condition	Description of characteristics assessed
0 - 1	Normal/ no effect	1. Normal oocytes with surrounding perifollicular cells 2. Normal ovary with all stages of oocyte development 3. Mature oocytes in the lumen of the ovary
1 - 2	Minimal effect	1. Ovum lamellae and follicular lining intact 2. Increase vacuolated oocytes 3. Perifollicular cell hypertrophy 4. Decrease in the number of developing oocytes
2 - 3	Moderate	1. Increased oocytes atresia 2. Hyperplasia of the follicular envelope/ epithelium characterized by increase in number and size of granulosa and theca cells. 3. Degeneration of the stroma 4. Vacuolation.
4 - 4	Severe effect	1. Granulomatous inflammation

	2. Degradation or atresia of the oocytes characterized by nuclear fragmentation and clumping of the chorion
	3. Egg debris in the oviduct
	4. Interstitial fibrosis
	5. Decreased vitellogenesis which is characterized by absence of yolk material in oocytes

Table 3.9 Grading criteria for the evaluation of the histopathological effect of pawpaw seed meal on the liver tissues of *O. mossambicus* (adapted from USEPA, 2006; OECD 2009).

Grading	Condition	Description of characteristics assessed
0 - 1	Normal/ no effect	1. Normal architecture with hepatic portal vein and hepatic artery
		2. Normally arranged Hepatocytes separated by sinusoids
		3. Reticulin fibres supporting both hepatocytes and sinusoids
1 - 2	Minimal effect	1. Increased hepatocytes basophilia
		2. Increasing Vacuolation of hepatocytes
		3. Dilation of sinusoid
2 - 3	Moderate	1. Decreased hepatocytes basophilia
		2. Onset of hepatic degeneration
		3. Vacuolation of hepatocytes
		4. Proliferation of sinusoid
		5. Hypertrophy of the hepatocytes
4 - 4	Severe effect	1. Shrunken hepatocytes and hepatic degeneration
		2. Vacuolation of hepatocytes
		3. Hepatocytes with picnotic nuleus
		4. Necrosis of the hepatocytes

Severity grading of the histopathology

Phytochemicals may exert either severe or subtle effects on the tissues. Estimation of the severity or degree of pathology exerted on the tissue is somewhat semi-quantitative. Severity grading compares changes exerted on the animal among treatment groups, the changes can be discrete or diffused.

 Severity grading according to USEPA, (2006)include;

1) Grade O: No effect

2) Grade 1: Minimal effect of which a particular discrete effect occurs once or twice in a microscopic field. The effect is also classified as minimal if less than 20% of the tissue is involved in case of a diffuse alteration of the tissues.

3)   Grade 2: Mild effect in which 3 to 5 of a particular discrete effect occur per microscopic field or 30 to 50% if the effect is diffuse.

4)   Grade 3: Moderate if there is 6 to 8 occurrence of discrete and/or 60 to 80% of diffuse effects.

5)   Grade 4: Severe effect if more than 9 occurrence (discrete) or more than 80% of the tissues in diffusely distributed alteration.

3.7   Statistical analysis

Morphometric parameters (total length, standard length, and body weight) of the experiment 1, 2 and 3 measured were subjected to a single factor analysis of variance (ANOVA) to ascertain the effect of the inclusion of the PSM in the diet of *O. mossambicus* on the parameters. Variant means were separated using a Bonferroni (Dunn) t test and mean differences with p<0.05 was considered statistically significant.

The derived indices (condition factor, specific growth rate, and survival rate) in experiment 1 and 2 were subjected to a single factor analysis of variance (ANOVA) to ascertain the effect of the inclusion of the PSM in the diet of *O. mossambicus* on the indices. Variant means were separated using Bonferroni (Dunn) t test and mean differences with p<0.05 was considered statistically significant

Significant deviation in sex ratio was analysed using Logistic regression and Chi- square test to ascertain the level of deviation from the expected 1:1 (M:F) ratio.

Statistical analysis of the experiment 1 data was performed using SAS system ('local', X64_7PRO, Version 9.1). The results are presented as mean±SE. The data were analysed by a one way analysis of variance (ANOVA) with a confidence interval of 95%. Variant means were separated using a Bonferroni (Dunn) t test.

Data from blood analysis

The sex hormone (17β- estradiol and 11-ketotestosterone), blood chemical parameters (cholesterol, total protein, albumin, globulin, glucose and albumin/ globulin ratio) and the haematological parameters (RBC, HGB, HCT, MCV, MCH, MCHC, RDW, PLT, WBC, NEU, LYM, MONO, EOS, and BASO: please see appendix), assayed for experiment 2 and 3. The data were analysed by a one way analysis of variance (ANOVA) with confidence interval of 95% to determine the effect of PSM on the parameters measured. Variant means were separated by using a Bonferroni (Dunn) t test. Statistical analysis of experiment 2 and 3 were performed using the XLSTAT software program (version: 2015.2.02.18165).

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Chapter 4

The effect of Pawpaw (*Carica papaya*) seed powder on the sex differentiation, growth and survival of *Oreochromis mossambicus* fry

Abstract

The production of monosex Mozambique tilapia (*Oreochromis mossambicus*, OM) populations is a potential and effective solution for the precocious breeding and indiscriminate spawning that occur in mixed sex Mozambique tilapia culture systems, which in turn result in the overcrowding of ponds and stunted growth. This study investigated the possibility of using Pawpaw (*Carica papaya*) seed meal, (PSM) to skew the sex ratio of sexually undifferentiated OM fry of 10.02±0.14 mm mean total length to obtain all-male populations. A total of 2160 OM were used in the study, and fish were allocated to 12 treatment groups, with 60 fish per treatment group. The experimental diets fed in the study included a basal diet (BD; negative control), 60mg 17α-methyltestosterone/kg BD (positive control), and 5 treatment diets containing 10, 15, 20, 25 and 30g PSM/kg of the BD, respectively. The experimental diets were fed to the treatment groups for 30 days, and 120 days, respectively. At an inclusion level of 10g/kg of basal diet, PSM was able to skew the sex ratio in favour of males (60% males vs. 40% females). The proportion of males to females increased with an increase in PSM content of the diet. The maximum masculinization effect was observed when 20g PSM/kg BD was fed, with male to female ratio for the groups that received 30g PSM/kg BD not being significantly different. The highest percentage phenotypic males (77.8%) induced by the PSM was obtained among in the groups that received 20g and 30g PSM/kg BD. The duration of feeding the PSM treatment diets for longer than 30 days did not influence the male to female ratio in any of the treatment groups. The growth and survival rates of the fish were not influenced by any of the treatment diets. Gonad abnormalities that were observed when 30 g PSM/kg BD were fed included mild hyperplasia of the follicular epithelium and atresia of the follicles in female fish, and hypertrophy of the interstitial cells and mild vacuolization of the germ cells was observed in male OM. Pawpaw seed meal resulted in degeneration of hepatic tissue, dilation of sinusoid tissue, and vacuolization of hepatocytes. The study demonstrated that an inclusion level of 20g PSM/kg BD and higher was effective in producing a high percentage of male OM populations, which in turn will result in more uniform growth rates, and an optimal size at harvest to ensure the viability and cost-efficiency of OM production systems.

4.1 Introduction

Tilapia, endemic to Africa, is a species of choice in aquaculture systems, for they can tolerate a wide range of culture conditions. Their adaptability to a wide range of culture conditions allow this species to be exploited as

a food fish for the increasing population of Sub-Saharan Africa. However, cost-efficient production is hampered by the precocious maturation and indiscriminate spawning of most tilapia species, which in turn results in overpopulation and consequent stunted growth and production losses (Varadaraj & Pandian, 1990; Toguyeni *et al.*, 2002). The concept of mono-sex culture production of tilapia presents an opportunity to overcome the limitation of precocious breeding, and it is further supported by the hypothesis that male tilapia grows faster than females (Otubusin, 1988; Davis *et al.*, 2010). The establishment of all-male tilapia populations can also prevent the problem of undesired indiscriminate spawning, which will contribute to minimizing stunted growth, and thus optimizing the time to harvest.

Various methods are available for the production of all-male tilapia populations, and include manipulation of the immediate environment of the animal (heat or cold shock), manual separation of sexes, genetic engineering ("super male" tilapia), and hormonal induction. In gonochoristic fish like tilapia, phenotypic sex reversal can only be achieved in young fish with undifferentiated gonads (Baras *et al.*, 2000). The most common commercially viable option in sex reversal in tilapia is by the use of steroid hormones that are administered during the undifferentiated stage of gonadal development. Steroid hormones play a vital role in sex differentiation in tilapia (Rothbard *et al.*, 1987) and 17 α- methyl testosterone, a synthetic androgen when incorporated into the diet of sexually undifferentiated tilapia at an appropriate dosage and period, can convert genetic females to phenotypic males (Johnstone *et al.*, 1983; Ridha & Lone, 1990; Pandian & Sheela, 1995). Despite the effectiveness of the synthetic hormone in sex reversal and subsequent increase in overall fish production, concerns have been expressed concerning the health implication of the use of 17α-metyltestosterone in food fish production. Apart from the suspected health hazards of employing synthetic hormones in food fish production, concerns have also been expressed about the fact that it is expensive and difficult to obtain (Jegede, 2010a). An alternative approach is to use natural substances that will pose no environmental risk or health risk to humans, and which can manipulate the gender to obtain all-male tilapia populations.

Carica papaya (pawpaw) is a widely grown fruit tree in the tropics and subtropics. It belongs to the family Caricaceae, and according to studies, its fruit contain protein, fat, carbohydrates, vitamins A and C, carotene, minerals (calcium, iron and sodium), and fibre (Adebiyi *et al.*, 2002; Alobo, 2003; Krishna *et al.*, 2008). The different parts of the plant, including the seeds, are reported to possess antibacterial, antifungal, pesticidal, and anti-fertility properties (Oliver-Bever, 1982; Eno *et al.*, 2000; Doughari *et al.,* 2007; Mansura *et al.*, 2009). It has also been credited with molluscicidal activities against freshwater snail *Lymnaea acuminata* (Jaiswal & Singh, 2008). Pawpaw has also been used as an anti-fertility agent in several animal models (Joshi & Chinoy, 1996; Adebiyi *et al*., 2003; Manivannan *et al.*, 2004; Verma *et al*., 2006). The seeds, when included in the diet of rats,

68

are reported to have an anti-implantation effect in female rats, and result in sterility in the males (Das, 1980). Ampofo-Yeboah (2013) investigated the potential of pawpaw seed meal, when included as part of a basal tilapia diet, to produce monosex tilapia populations.

There are few reports on the effect of pawpaw seed meal (PSM) on fish reproduction. Several studies investigated its effect on the gonado-somatic index, hatchability, egg diameter and fecundity, and the architectural integrity of the reproductive organs of sexually mature fish (Jegede and Fagbenro, 2008; Hossam and Wafaa, 2011; Abdelhak *et al*., 2013). The only report to date on the use of pawpaw seed meal for the production of monosex tilapia populations was by Ampofo-Yeboah (2013). In the study, Ampofo-Yeboah (2013) achieved a 65% masculinization rate in Mozambique tilapia (*Oreochromis mossambicus*) at an inclusion of 15 g PSM /kg basal diet. Ampofo-Yeboah (2013) highlighted the need to determine the optimum inclusion level of PSM that will result in the maximum percentage of male tilapia from undifferentiated Mozambique tilapia fry. The liver plays an important role in fish reproduction as it is the organ that secrets the yolk precursor vitellogenin and also in the neuroendocrine system of fish. Because of the importance of liver in reproduction, the impact of PSM on the histological architecture of the organ has been studied in *O. niloticus* (Hossam & Wafaa, 2011), however there was no available literature presently on the effect of this phytochemical on the liver function and immune system of *O. mossambicus*.

The aim of this study was to determine the optimal inclusion level of PSM in the diet of undifferentiated Mozambique tilapia fry to obtain all-male populations. To assess whether the phytochemicals will affect the normal functioning of the liver, as well as compromise the immune competence of treated fish, the effect of PSM on the growth and survival, and architectural integrity of the liver and the reproductive organs were also assessed.

4.3 Materials and methods

4.3.1 Experimental location and facilities

The experiments were carried out in the water recirculation unit of the Aquaculture Research facilities based at the Welgevallen Experimental Farm of Stellenbosch University, South Africa. The re-circulatory aquaria system consists of 72 glass aquaria tanks, each with a dimension of 57x53x40cm (LxWxH), and a capacity of 120.8L. Aeration and filtration components are incorporated into its design to ensure the maintenance of optimal culture conditions throughout the trails.

4.3.2 Experimental animals and husbandry

A total of 2160 yolk sac *O. mossambicus* fry were used in the study. The fish were obtained from the Rivendell hatchery, Grahamstown South Africa. The age of the experimental animals varied between one to two weeks old, and the average weight and total length of the fish was 0.036±0.014 g and 1.02±0.14 cm respectively.

Prior to stocking the respective tanks, 5 fry were randomly taken from each replicate, and the individual weight and total body length of each fish recorded. To avoid stocking a single progeny group in one treatment, fry were sorted and randomly allocated to each treatment, at a stocking density of 60 fish per replicate, and a total of 3 replicates per treatment.

The fish were fed *ad libitum* three times a day (9.00 − 9.30h, 13.00 − 13.30h and 17.00 − 17.30h) with the experimental diets. The waste and uneaten food in the aquaria were carefully removed daily by siphoning and the tanks refilled with fresh water. The culture tanks were checked daily for mortalities and the dead fish removed immediately.

The physicochemical parameters (temperature, dissolved oxygen and pH) of the culture water were monitored every other day. For more information on the specific parameters monitored, and the ranges recorded throughout the study, please refer to Chapter 3 for more details.

4.3.3 Experimental diets

Pawpaw seeds were obtained from ripe pawpaw fruit that were acquired from fruit vendors in Stellenbosch, South Africa. The fresh seeds were after removal from the fruit, processed to obtain a pawpaw seed meal (PSM), which was packaged in Ziploc bags, and stored in a cool dry environment until later use. For more information on the processing of the pawpaw seed and the preparation of the PSM, please refer to Chapter 3.

The standard (basal) diet used throughout the study consisted of a commercial tilapia diet (Nutroscience (Pty) Ltd, Malmesbury, South Africa). The composition of the basal diet is presented in Chapter 3. The respective experimental diets are presented in Table 4.1.

4.3.4 Experimental layout

There were 12 experimental treatment groups based on the inclusion level of PSM and three replicates for each treatment. The treatment groups and their designation are indicated in Table 4.1.

Table 4.1 Inclusion level of pawpaw seed meal (PSM) and designation of treatment groups.

Treatment	Inclusion level	Designation
Basal diet (BD; negative control) for 4 months	No inclusion of PSM	BDM4
BD + 60mg Methyl testosterone for 1 month	No inclusion of PSM	MTM1
BD + PSM for 1 month	10g PSM/ kg BD	P10M1
	15g PSM/ kg BD	P15M1
	20g PSM/ kg BD	P20M1
	25g PSM/ kg BD	P25M1
	30g PSM/ kg BD	P30M1
BD + PSM for 4 months	10g PSM/ kg BD	P10M4
	15g PSM/ kg BD	P15M4
	20g PSM/ kg BD	P20M4
	25g PSM/ kg BD	P25M4
	30g PSM/ kg BD	P30M4

Sampling frequency

Sampling and taking of the morphometric of a representative sample in each replicate was undertaken every two weeks and the sample size was 10 fish per replicate. The final sampling and taking of the morphometric of a representative sample in each replicate was undertaken at the 120th day and the mortality in each treatment was recoded so as to calculate the survival rate.

4.3.5 Data recorded

Length – weight measurement – The total and standard lengths of the specimen were measured using a measuring board graduated in centimeters. The total length was measured from the anterior most extremity of the fish to the end of the tail fin to the nearest centimeters. The standard length was measured from the most anterior extremity to the base of the tail fin. The base was identified by a crease when the tail was bent sharply from side to side. Top loading balance (Electronic Balance, UWE, HGS-300, capacity: 300 x 0.01g, Serial # P9440) was used to measure the body weight of the fish samples to the nearest grammes. The mortality in different treatment groups were recorded also the number of females and males at the end of the experimental period.

Condition factor, growth and mortality rates were determined.

The condition factor (K) of each fish was calculated using the formula:

$$K = \frac{100W}{L^3} \quad \text{(Ricker, 1975)}$$

Where L = standard length (cm) and W = Body weight (g)

Specific growth rate was calculated using the formula:

SGR = $\underline{LnW_f - LnW_i}$ X100

        T (days)   (Tran-duy *et al*., 2008)

Where W_f = final weight and W_i = initial weight

Survival rate was calculated using the formula:

                          Final number of fish

Survival rate = ---X100

                            Initial number of fish

4.3.6 Histological evaluation of tissue samples

At the end the experimental period, histology of the liver and gonads were undertaken to determine the effects of the phytochemicals on their architectural integrity. Gonad and liver tissue samples were collected from 4 fish (two males and two females) and 2 fish (1 male and 1 female), respectively from each replicate, and preserved in 10% buffered formalin until further processing.

Processing of the slides for histological examination was performed according to standardized protocols at the Department of Physiology, University of Stellenbosch, South Africa. For more information on the imbedding and staining procedures, please refer to Chapter 3.

After the slides were mounted, all slides were graded according to a predetermined scale for gonad and liver integrity, respectively. The main grading categories for gonad integrity were based on a numerical scale in comparison with the control of this study using OECD (2009) as a guide for the description, please refer to Chapter 3 (Tables 3.6 and 3.7).

The main grading categories for liver function pathology were also based on numerical scale in comparison with the control of this study using OECD (2009) as a guide for the description. For a more detailed description of the different grading criteria, please refer to Chapter 3 (Tables 3.8).

4.3.7 Statistical analysis

Morphometric parameters (total length, standard length, and body weight) measured were subjected to single factor analysis of variance (ANOVA) to ascertain the effect of the inclusion of the PSM in the diet of *O.*

mossambicus on the parameters. Variant means were separated using Bonferroni (Dunn) t-test and mean differences with p<0.05 was considered statistically significant.

The derived indices (condition factor, specific growth rate, and survival rate) in experiment 1 and 2 were subjected to a single factor analysis of variance (ANOVA) to ascertain the effect of the inclusion of the PSM in the diet of *O. mossambicus* on the indices. Variant means were separated using Bonferroni (Dunn) t-test and mean differences with p<0.05 was considered statistically significant. Sex ratio among different treatments was calculated. Significant deviation in sex ratio was analysed using Logistic regression and Chi- square test to ascertain the level of deviation from the expected 1:1 (M:F) ratio.

All statistical analysis was performed using the SAS software programme ('local', X64_7PRO, Version 9.1).

4.4 RESULTS

4.4.1 Morphometric parameters

At the termination of the trial in week 16, the mean total length of P25M1, P10M4, P30M4 and BDM4 were significantly shorter than that of P20M4 while the mean weight of P25M1, P10M4 and BDM4 were also significantly lighter than that of P20M4. Also the Fulton's condition factor of P20M4, P25M4 were significantly higher than that of P10M1, P10M4 and P15M4 as shown in Table 4.2.

Table 4.2 Morphometric parameters (mean ± SD) of *O. mossambicus* fed diet containing pawpaw seed meal (PSM) during a 120 day culture period.

Treatment	Total length (cm)	Standard length (cm)	Weight (g)	Condition factor
BDM4 (control)	11.82 [c] ±1.04	9.44 [b] ±0.86	34.52 [b] ±7.94	4.06 [ab] ±0.39
MTM1 (control)	12.49 [ab] ±0.71	10.02 [ab] ±0.62	39.57 [ab] ±5.66	3.94 [ab] ±0.45
Period 30 days				
P10M1	12.24 [ab] ±1.41	9.98 [ab] ±1.25	38.88 [ab] ±11.32	3.87 [a] ±0.69
P15M1	12.2 [ab] ±0.91	9.78 [ab] ±0.78	37.68 [ab] ±7.82	3.99 [ab] ±0.28
P20M1	12.31 [ab] ±1.23	9.81 [ab] ±1.02	38.68 [ab] ±9.42	4.03 [ab] ±0.35
P25M1	11.99 [b] ±1.03	9.54 [b] ±0.82	34.58 [b] ±8.83	3.93 [ab] ±0.34
P30M1	12.17 [ab] ±0.98	9.81 [ab] ±0.81	38.13 [ab] ±8.03	3.99 [ab] ±0.28
Period 120 days				
P10M4	11.74 [b] ±0.94	9.35 [b] ±0.76	35.11 [b] ±8.29	3.88 [a] ±0.3
P15M4	12.07 [ab] ±1.11	9.6 [ab] ±0.91	37.23 [ab] ±8.91	3.88 [a] ±0.31
P20M4	12.91 [a] ±0.95	10.33 [a] ±0.69	43.2 [a] ±9.04	4.24 [b] ±0.51
P25M4	12.32 [ab] ±0.87	9.92 [ab] ±0.7	38.04 [ab] ±7.0	4.15 [b] ±0.3
P30M4	11.84 [b] ±1.0	9.56 [b] ±0.79	36.08 [ab] ±8.77	4.07 [ab] ±0.36

[a, b] Columns with different superscripts differ significantly (P < 0.05)

4.4.2 Effect of Pawpaw seed meal on sex ratio

The identification of gender by means of the absence or presence of their gonads could be performed with ease at the end of the 120-day trial period. The potential of pawpaw seed meal included as part of the basal tilapia diet, and of the 17 α -methyl testosterone to masculinize tilapia is presented in Table 4.3.

Table 4.3 The influence of pawpaw seed meal and 17α-methyl testosterone, included as part of a basal tilapia diet, to masculinize *O. mossambicus* fry after a 30 day and 120 day treatment period, respectively.

Treatment	N	% Males	%Females	M:F	X^2	P-value
BDM4 (control)	90	52.22	47.78	1.09: 1[a]	0.21	0.65
MTM1 (Control)	90	83.33	16.67	5: 1[b]	23.16	<0.0001
Period 30 days						
P10M1	90	60	40	1.5: 1[a]	2.69	0.10
P15M1	90	63.33	36.67	1.72: 1[b]	4.37	0.037
P20M1	90	74.44	25.56	2.91: 1[b]	13.03	0.000
P25M1	90	74.44	25.56	2.91: 1[b]	13.03	0.000
P30M1	90	77.78	22.22	3.5: 1[b]	16.54	<0.0001
Period 120 days						
P10M4	90	62.22	37.78	1.65: 1[a]	3.77	0.052
P15M4	90	62.22	37.78	1.65: 1[a]	3.77	0.052
P20M4	90	77.78	22.22	3.5: 1[b]	16.54	<0.0001
P25M4	90	74.44	25.56	2.91: 1[b]	13.03	0.000
P30M4	90	75.56	24.44	3.09: 1[b]	14.16	0.000

[a, b] Columns with different superscripts differ significantly (P < 0.05)

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In the negative control group (BDM4) which were fed the basal diet only, the male to female (M: F) sex ratio of 1.09: 1 did not differ significantly with the expected sex ratio of 1: 1, however there were significantly higher number of males to females among the positive control group (MTM1) which were fed diet containing 60mg methyl testosterone per kg of the basal diet for 30 days. In all the treatment groups, the highest sex ratio of five males to a female (5: 1) was obtained in 17α-methyl testosterone treated group (MTM1). At an inclusion level of 10 g/kg of basal diet, PSM was able to skew the sex ratio in favour of males (60% males to 40% females) as shown in Table 4.3.
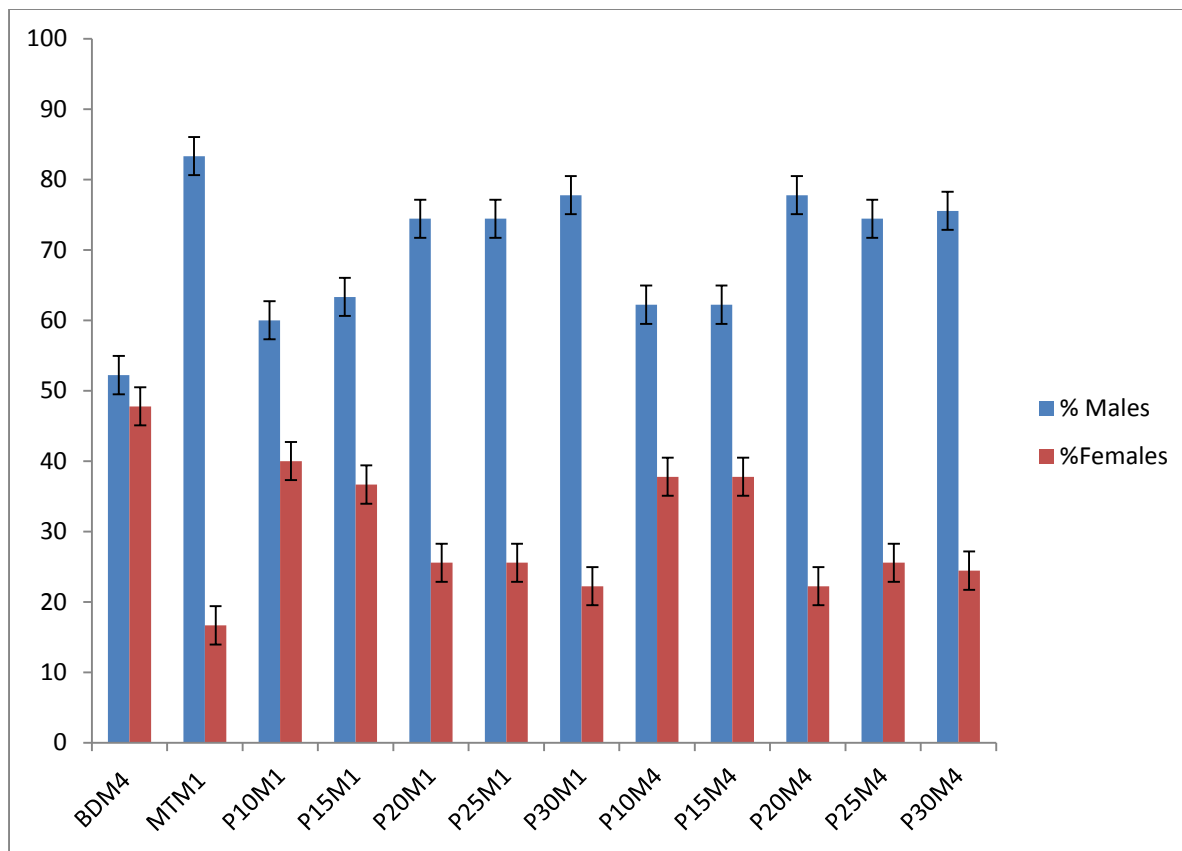
Figure 4.1 The sexual phenotype of *O. mossambicus* fed diet containing pawpaw seed meal (PSM) for 30 and 120 days.

The proportion of males increased with an increasing dosage of PSM, with the maximum masculinization achieved at an inclusion level of 20 g/kg BD and 30 g/kg BD resulting in 77.8% males produced (Figure 4.1). When the masculinization success of the diets obtaining at 20 g/kg BD and 30 g/kg BD respectively, was compared, no significant differences were observed in terms of the numbers of males produced (Table 4.3).

When the masculinization success was also compared in terms of the duration of the feeding regimes of one and four months, no significant differences were observed in terms of the number of males produced Table 4.3).

4.4.3 Effect of Pawpaw seed meal on specific growth rate
When the specific growth rate (SGR) of different treatment groups was compared, P20M4 had a significantly higher SGR than BDM4 (control), P10M4 and P25M1 as shown in Table 4.4

Table 4.4 Specific growth rate of *O. mossambicus* fed diet containing pawpaw seed meal (PSM) during a 120 day culture period.

Treatment group	Initial weight	Final weight	SGR
BDM4 (control)	0.036±0.016	34.52±7.94	3.54[b]
MTM1 (control)	0.04±0.015	39.57±5.66	3.68[ab]
Period 30 days			
P10M1	0.035±0.017	38.88±11.32	3.66[ab]
P15M1	0.035±0.012	37.68±7.82	3.63[ab]
P20M1	0.037±0.014	38.68±9.42	3.66[ab]
P25M1	0.036±0.014	34.58±8.83	3.54[b]
P30M1	0.036±0.017	38.13±8.03	3.64[ab]
Period 120 days			
P10M4	0.038±0.017	35.11±8.29	3.56[b]
P15M4	0.038±0.017	37.23±8.91	3.62[ab]
P20M4	0.039±0.014	43.2±9.04	3.77[a]
P25M4	0.036±0.013	38.04±7.0	3.64[ab]
P30M4	0.037±0.019	36.08±8.77	3.59[ab]

[a, b] Columns with different superscripts differ significantly ($P < 0.05$)

4.4.4 Effect of pawpaw seed meal on survival

The inclusion of pawpaw seed meal in the diets did not affect the survival in most of the treatments. The exception was treatment P30M1, where a higher mortality ($P<0.5$) occurred due to a physical blockage of one of the water inlets, which resulted in the aeration system to temporarily stop functioning. However, there was no distinct trend in the survival rate of different treatment groups that can be attributed to the effect of the experimental diet as shown in Table 4.5.

Table 4.5 Survival rates of *O. mossambicus* fed diet containing pawpaw seed meal (PSM) during a 120 day culture period.

Treatment	Num. stocked	Num. Harvested	Mortality	Survival (%)
BDM4 (control)	180	162	18	90.00[a]
MTM1 (control)	180	161	19	89.44[a]
Period 30 days				
P10M1	180	165	15	91.67[a]
P15M1	180	140	40	77.78[bc]
P20M1	180	136	44	75.56[bc]
P25M1	180	140	40	77.78[bc]
P30M1	180	124	56	68.89[c]
Period 120 days				
P10M4	180	153	27	85.00[ab]
P15M4	180	153	27	85.00[ab]
P20M4	180	139	41	77.22[bc]
P25M4	180	138	42	76.67[bc]
P30M4	180	141	39	78.33[bc]

[a, b] Columns with different superscripts differ significantly (P < 0.05)

4.4.5 Histopathological changes induced by Pawpaw seed meal

Effect on the ovary

The pathological effect observed in the ovary includes fragmentation of the ovarian nuclei, hyperplasia of the follicular epithelium and atresia of the follicles. The effect on ovary were observed in those fish from treatment group P30M1 fed diet containing 30g of PSM/ kg of BD in which out of the six fish examined two were affected. Also in treatment groups P25M4 and P30M4 that were fed 25 and 30 g respectively of PSM/ kg of the basal diet: 2 fish out of the 6 sampled were affected in group P25M4 while three in group P30M4.

Effect on the testes

The histopathological effect observed in the testis includes hypertrophy of the interstitial cells and mild vacuolation of the germ cells. One each of the six fish examined in the treatment groups P25M1, P30M1 and P25M4 were affected while two of the six fish examined in group P30M4 had the histopathological lesions in the testis.

78

Effect on the liver

The pathological lesions observed in the liver include vacuolization of hepatocytes, dilation of sinusoid and hepatic degeneration. One of the six fish examined in treatment group P25M1 was affected while two of the fish examined in groups P30M1, P25M4 and P30M4 had the pathological lesions in their liver tissues (Table 4.6).

Table 4.6: Severity grading of the histological changes of the gonads and liver of *O. mossambicus* fed pawpaw seed meal (PSM) for 30 and 120 days.

Treatment group	Effect on testes		Effect on ovary		Effect on the liver	
	Number examined	Number affected	Number examined	Number affected	Number examined	Number affected
P10M1	6	0	6	0	6	0
P15M1	6	0	6	0	6	0
P20M1	6	0	6	0	6	0
P25M1	6	1	6	0	6	1
P30M1	6	1	6	2	6	2
P10M4	6	0	6	0	6	0
P15M4	6	0	6	0	6	0
P20M4	6	0	6	0	6	0
P25M4	6	1	6	2	6	2
P30M4	6	2	6	3	6	2
BDM4	6	0	6	0	6	0
MTM1	6	0	6	0	6	0

79
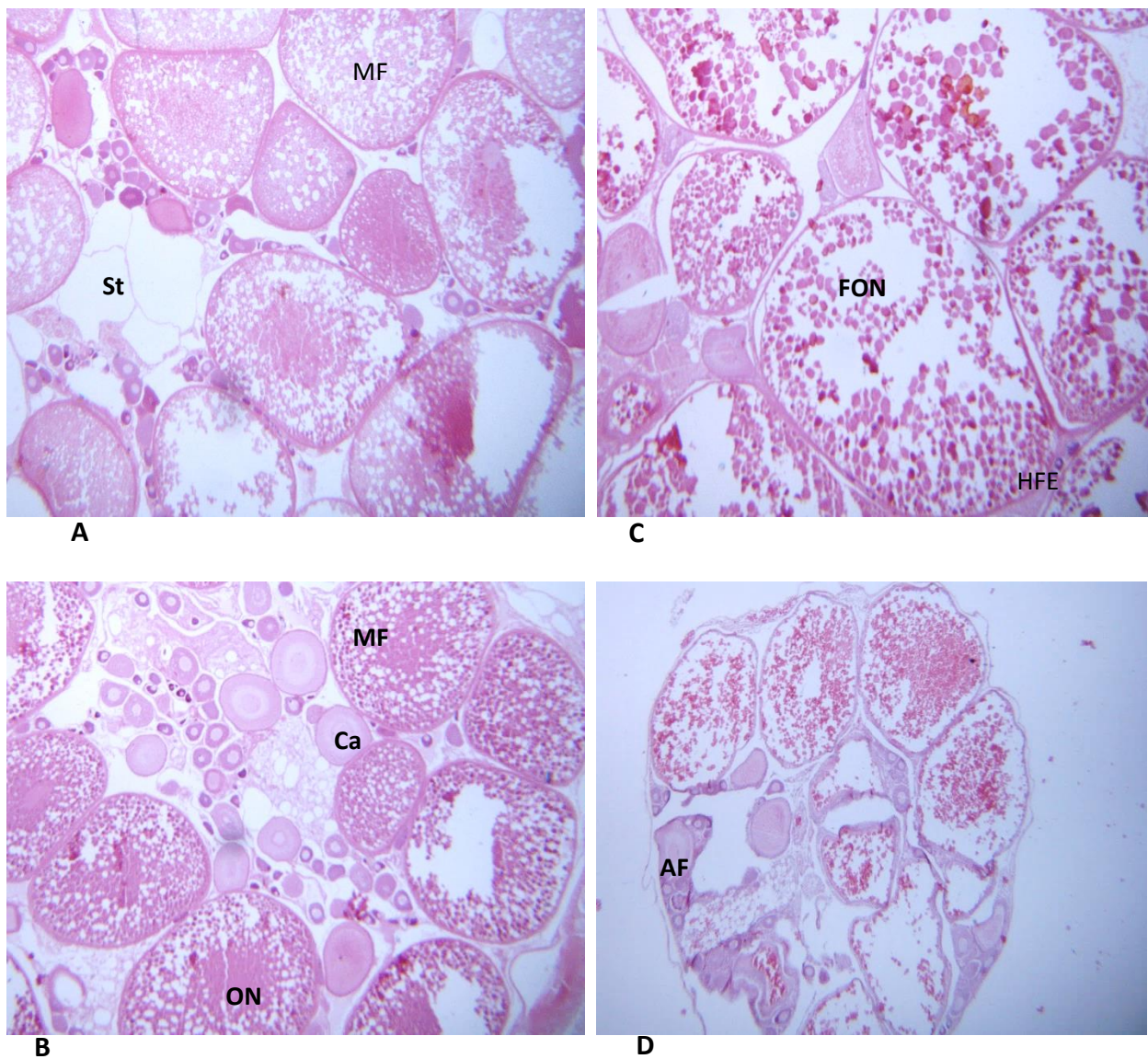
Plate 4.1 (A – D): *Oreochromis mossambicus* ovary showing the effect of PSM on the ovarian tissues (H&E staining, 400X magnification). A and B represent normal ovarian tissues with follicles at various stages of development, while C and D represent ovarian tissues with certain pathological changes. MF – mature follicle; Ca – capsule; ON – ovarian nucleus; St – stroma; AF – atretic follicle; HFE – hyperplasia of follicular epithelium; FON – fragmentation of ovarian nucleus.
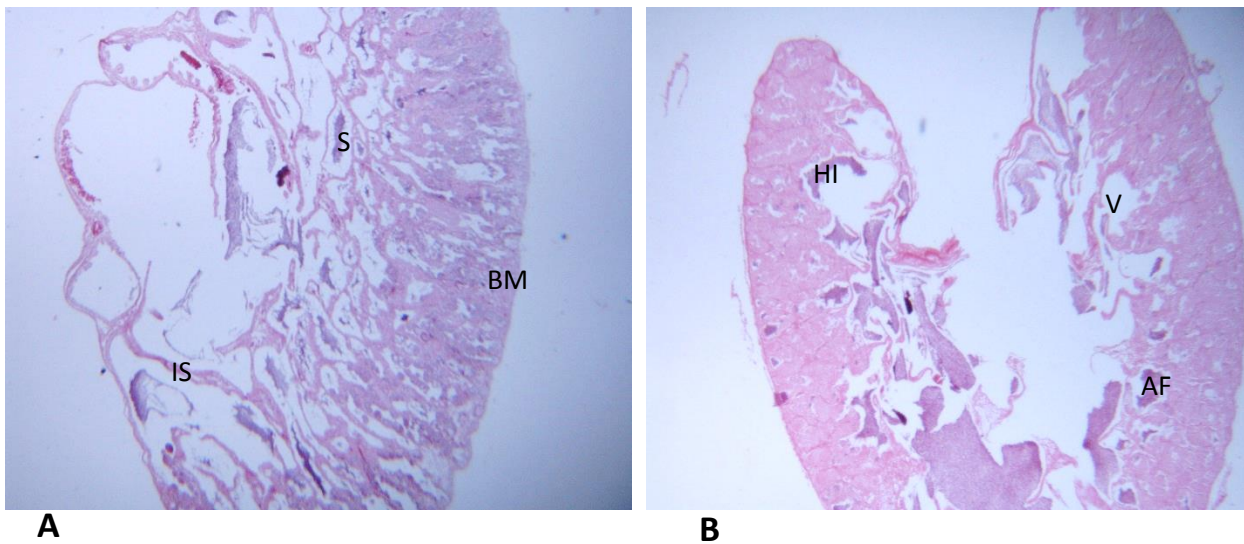
Plate 4.2 (A – B): An *Oreochromis mossambicus* testis showing the effect of PSM on the testicular tissues (H&E staining, 400 X magnification). A represents normal testicular tissue, while B represents testicular tissue from fish fed PSM. S – spermatocyte; IS – interlobular septa; BM – basement membrane; HI – hypertrophy of interstitial cells; AF – atresia of follicles; V - vacuolization of germ cells.
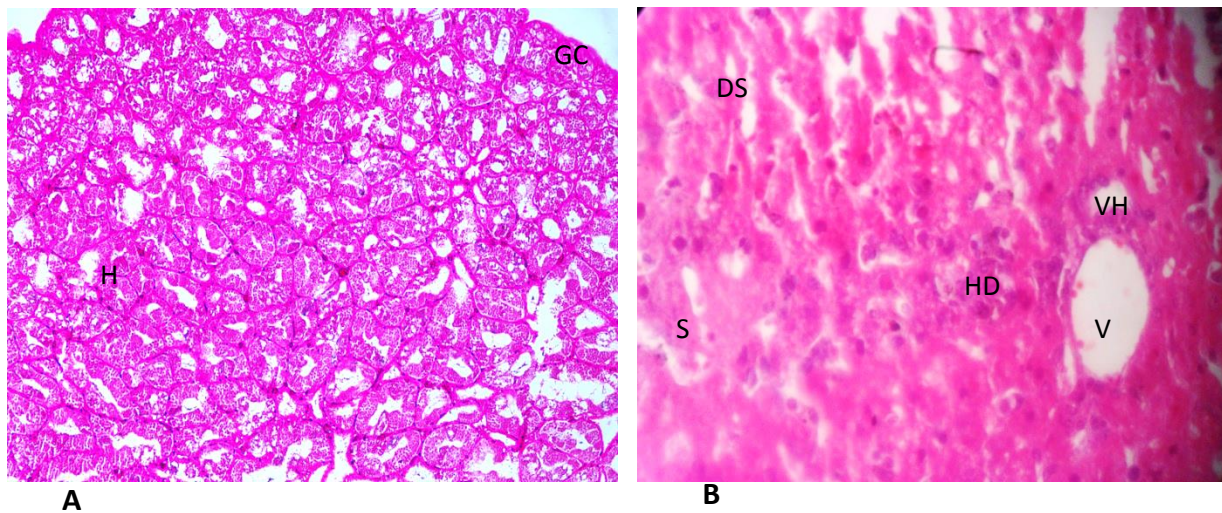
Plate 4.3(A – B): *Oreochromis mossambicus* liver showing the effect of PSM on the hepatic tissues. A represents normal hepatic tissue, and B represents hepatic tissue from fish fed PSM. H – hepatocytes; S – sinusoid; GC – Glisson's capsule; V – hepatic venule; VH – vacuolization of hepatocytes; HD – hepatic degeneration; DS – dilation of sinusoids.

4.5 DISCUSSION

The male: female ratio 1.09: 1 achieved for the negative control group (BDM4) that received the BD in this study did not deviate significantly from the expected 1: 1 (M: F) ratio. In this study, the diets that contained PSM resulted in the masculinization of the treated animals, which was in agreement with the observation of Ampofo-Yeboah (2013). An inclusion rate of 10g PSM/kg BD fed for 30 and 120 days, respectively, resulted in a M: F ratio of 1.5:1, which did not differ significantly from that obtained for OM fry that received the basal diet during the same two intervals.

The percentage of males increased with an increase in the PSM contents of the diets, with the highest degree of masculinization observed with the diets containing 20 g PSM/kg BD. An inclusion level of 20 g PSM/kg BD resulted in 77.8% males compared to 22.2%, which equates a M: F ratio of 3.5:1. The percentage males achieved by the highest inclusion level of 30 g PSM/kg BD did not differ from that achieved with the 20g PSM/kg BD. The maximum percentage of sex reversed fish (77.8%) differed considerably from a 65% masculinization rate that was reported by Ampofo-Yeboah (2013) for *O. mossambicus*. Ampofo-Yeboah (2013) using 15g of PSM/kg of BD achieved 65% male brood, however when the dosage was increased to 20g PSM /kg of BD and above, 77.8% masculinization was obtained. The fact that an inclusion level of 20 g PSM/kg BD (treatment group P20M4) resulted in 22.2% female fish, warrants further investigation to determine which physiological mechanisms may prevent a complete masculinization of treated fish. The occurrence of female fish even after treatment still present an opportunity for these fish to reproduce precociously, which will result in the overcrowding of pond systems to occur. There was no significant difference in the number of males produced during the 120 day treatment period, when compared to fish that received the treatment diets for the 30 day treatment period. This can be ascribed to the fact that sex reversal takes place during the period of sex differentiation, which takes place between 10 – 20 days post hatch *in O. mossambicus* (Varadaraj & Pandian, 1987). Results from this study indicated that a prolonged treatment with PSM, i.e. for an interval exceeding the period of sex differentiation, did not result in a higher percentage of males obtained. This is supported by the findings of Kwon *et al*. (2000), who reported that masculinization occurs 7 – 37 days post-hatch in *O. niloticus* fry.

Different techniques have been employed in the production of mono-sex male tilapia, the most popular of which is the use of steroid hormones to skew the sex ratio in favour of males. The hormone 17α-methyltestosterone (MT) is the hormone of choice to stimulate masculinization of tilapia species (Pandian & Kirankumar, 2003; Haniffa *et al*., 2004; Babiak *et al.*, 2012). In this study, the positive control which consisted 60 mg MT/kg BD resulted in 83.3% males and 16.7% females, i.e. a M: F ratio of 5: 1. In practical tilapia farming, sex reversal of

less than 95% may not be the ideal since as little as 5% females in a culture facility can reproduce indiscriminately to the extent of overpopulating the system, thereby thwarting the effort made in the mono-sex production in the first place. The fact that even the inclusion of 60 mg MT/kg BD did not result in a 100% masculinization in this study, can potentially be ascribed to feed competition, which resulted in some individuals having less access to the treatments diet due to a dominance hierarchy experienced in the culture system (Varadaraj & Pandian, 1987).

Another reason for the less than 100% masculinization may be due to paradoxical feminization. Methyl testosterone which is a synthetic androgen, can be converted by means of the cytochrome P450 enzyme to form estrogen, which is a feminizing hormone (Afonso *et al.*, 2001). The aromatization of especially an excess of androgen to estrogen can paradoxically cause the feminization instead of the desired masculinization in some fish. Other factors that can influence the percentage of masculinization include water quality parameters such as temperature, pH, and dissolved oxygen of the culture system (Varadaraj *et al.*, 1994). Of these factors, water temperature is considered to be the most effective environmental factor in sex reversal (Baroiller & D'Cotta, 2001). It is suggested that high temperatures decrease aromatase activity, thereby predisposing to masculinization (D'Cotta *et al.*, 2001). In the present study, the physico-chemical parameters recorded during the experimental period showed that water temperature, dissolved oxygen (DO) and pH varied between 22.2 – 26.7 °C (mean±SD: 24.48±1.46 °C), 3.2 – 6.1mg/L (mean±SD: 4.98±0.73mg/L) and 6.12 – 6.72 (mean±SD: 6.42±0.18), respectively. These parameters were within the tolerable limit for fish culture (Timmons & Losordo, 1994). The water temperature in our study was potentially suboptimal to effectively allow for the sex reversal to take place. This hypothesis is supported by the findings of Uchida *et al.* (2004). In their study, that an aromatase inhibitor fadrazole resulted in 0%, 68.8& and 100% masculinization of zebra fish (*Danio rerio*) at water temperatures of 28.5 °C, 35 °C and 37 °C, respectively. If the water temperature of the experimental system were higher than the average of 24.48±1.46 °C recorded during the experimental period, this potentially could have contributed positively by resulting in a higher degree of masculinization in this study.

The morphometric parameters recorded at the termination of the experiment shows that the mean total length and mean weight of the 17α-methyl testosterone treated fish which had the highest male population was surprisingly low (12.49±0.71cm and 39.57±5.66g) compared to those of treatment group P20M4 that received 20 g PSM/kg of BD (12.91±0.95cm and 43.2±9.04g) although the difference was not statistically significant (p>0.05). However, mean weight of P20M4 was significantly higher than those of groups P10M4 (that were fed 10 g PSM/kg BD for 120 days), P25M1 (that were fed 25 g of PSM/kg of BD for 30 days) and BDM4 (that were fed the basal diet only for the entire duration of the experiment). Cone (1989) indicated that the relationship

83

between fish weight and length (i.e. condition factor) frequently used, compared the effect of biotic and abiotic factors on the health or well-being of fish. Plump fish may be indicators of favorable environmental conditions (e.g., good quality water, adequate nutrition and absence of disease condition), and thin fish may indicate less favorable environmental conditions. This is based on the hypothesis that heavier fish of a particular length are in a better physiological condition (Bagenal, 1978). In the presence of adverse environmental condition, every organism attempts to adjust itself for survival. It was observed in this study that the condition factor of P20M4, P25M4 were significantly higher than that of P10M1, P10M4 and P15M4 as shown in Table 4.2. Even though there were significant differences in weight and other morphometric parameters observed between some treatment groups, no dose response relationship was established in the present study.

The fact that the sex reversed fish in both the PSM and MT treated groups did not demonstrate any growth advantage over the untreated groups, was contrary to the report of Piferrer & Donaldson (1991), who recorded an improved growth rate of Coho salmon (*Oncorhynchus kisutch*) fed MT, they concluded from their study that androgen has an anabolic function in Coho salmon. However, there have been conflicting reports on the response of hormone-induced sex reversed fish on the growth. Whereas Park *et al.* (2004) and Toguyeni *et al.* (1997) opined that sex reversed fish grows faster than their mix sex cohorts, Goudie *et al.* (1994); Little & Edwards (2004) reported no significant growth between hormone treated and untreated ones. Tuan *et al*. (1998) was also of the opinion that whereas steroid hormones enhance the growth of genetic males, it suppresses that of the genetic females sex reversed to males. However, Little *et al*. (2003) reported that at prolonged nursing of 4 to 6 months, mono-sex tilapia attained larger individual size than their mixed sex counterparts even though there was no significant difference in survival rate. They concluded that increase in nursing period leads to a decrease in daily weight gain and specific growth rate in mixed sex tilapia due to reproductive activities. This opinion was collaborated by (Ridha & Lone, 1990) who observed no growth advantage in *Oreochromis spilurus* treated with 17α-methyl testosterone but after prolonged culture period of 243 days recorded significant increase in growth and SGR compared to the control.

A comparison of the growth rate of the different treatment groups in this study illustrated that the benefits of sex reversal in terms of enhanced growth and overall productivity were minor. It is assumed that the lack of well-established growth advantage expected in sex-reversed fish obtained in this study can potentially be ascribed to the fact that the fish had not been cultured for a prolonged period and in a more conducive environment like ponds, which will allow for a more pronounced effect in terms of growth. In practical aquaculture enterprise, sex reversal using exogenous hormones or phytochemicals are most often carried out in controlled environment such as aquaria, indoor concrete tanks or plastic containers. However after the treatment period, the sex

reversed fish are usually transferred to outdoor concrete tanks or earthen ponds which offer better environmental conditions for full expression of growth potential. If growth is the primary objective, longer culture periods thus present an opportunity for differences in performance to become evident. A prolonged culture period may present an economic advantage for the tilapia aquaculture industry if growth differences in relation to sex are fully exploited. The interest in the aquaculture industry for the production of mono-sex culture especially tilapia species was borne out of the assumption that male fish has a growth advantage over their female counterparts, except in such species as Atlantic halibut (*Hippoglossus hippoglossus* L) in which the culture of all female mono-sex is more economically advantageous since the females grow larger than the males (Hendry *et al*., 2003). Apart from the growth advantage ascribed to male tilapia, mono-sex culture is also practiced to mitigate the impact of indiscriminate spawning prevalent in the mixed culture system which predisposes to production of variable sized fish at harvest. Reduction of size variation at harvest enhances processing efficiency and economic value of the fish (Goudie *et al.*, 1994).

The survival rate of fish in this study ranged from 68.9% to 91.7%, and was the highest for the treatment group that received 10 g PSM/kg BD and the lowest for the treatment group that received 30 g PSM/kg BD. The respective treatment diets did not affect survivability of the treated fish, and were also not related to the dose or duration of the treatment. The fact that treatment group P30M1 had the least survival rate was not surprising since one of the replicate tanks of P30M1 recorded the high mortality rate due to aerating system failure when the inlet to the tank was clogged.

The effect of inclusion of PSM in the diet of sexually undifferentiated fry of *O. mossambicus* on survival was in agreement with the report of Ampofo-Yeboah (2013), who also reported no significant difference in the survival rates of PSM treated fish. However, there are contradictory reports on the effect of sex reversal on the survival rate of fish. Pandian & Sheela (1995) reported that hormonally sex reversed fish may suffer higher mortality and have a poor reproductive performance. Ridha & Lone (1990) also reported an inverse relationship between the dose of hormone and the survival rate of *Oreochromis spilurus*, indicating that sex reversal predispose fish to a lower survival rate. However, Jensen & Shelton (1979) reported higher survival rate in hormonally sex reversed fish than the untreated control. The fact that PSM did not predispose to mortality in this study shows that the phytochemical is well tolerated as a potential agent of sex reversal in *O. mossambicus* culture.

The inclusion of PSM in the treatment diets resulted in moderate changes in the architecture of the reproductive and hepatic organs. The pathologies observed in the ovaries included fragmentation of the ovarian nuclei, hyperplasia of the follicular epithelium, and atresia of the follicles. The pathology which though mild was

observed in fish fed high dose of the phytoestrogen including fish from treatment group P30M1 fed diet containing 30 g of PSM/ kg of BD and also in treatment groups P25M4 and P30M4 that were fed 25 and 30 g respectively of PSM/ kg of the BD. In the testis, the changes observed included hypertrophy of the interstitial cells, and mild vacuolization of the germ cells. Jegede & Fagbenro (2008) observed atretic follicles in the ovary, and increased interstitial cells and focal necrosis of testicular tissue in Nile tilapia (*O. niloticus*). Similarly Ekanem & Okoronkwo (2003) reported disintegrated sperm cells in Nile tilapia fed 4.9gPSM /kg feed/day. The histopathological lesions observed in the gonads in this study demonstrated that PSM, when administered at a dose higher than the optimum level required for masculinization and at a prolonged duration, can predispose the sex reversed fish to infertility.

Mild alteration in the normal architecture of the liver was also observed in this study, and they include dilation of sinusoids, vacuolization of hepatocytes, and general hepatic degeneration. There have been few reports of changes in the architecture of the liver caused by additives in fish diets. Velisek *et al*. (2009) reported degenerative hepatic cells in rainbow trout (*Oncorhynchus mykiss*) exposed to bifenthrin. Though the pathology observed in the liver in the present study were mild, it indicates that at a dose higher than the 30 g/kg of BD, PSM may predispose to liver damage which will invariably lead to poor egg quality and composition since the liver synthesizes yolk precursor vitellogenin. The fact that these lesions are minor and observed mainly in groups fed a high dose of the PSM for a prolonged period (i.e. 4 months) indicated a potential reversal of the phytoestrogenic effect of PSM on liver integrity on withdrawal of treatment.

4.6 CONCLUSIONS

This study suggests that an inclusion level of 20 g PSM/kg BD for 30 days treatment period is the optimal dosage to result in masculinization in *O. mossambicus*, with a 77.8% male population achieved. At this inclusion level the growth and survival of the fish were not negatively affected, and the minor histopathological lesions observed in the fish that received the higher doses and for a prolonged period, indicated that PSM was well tolerated. From the result of the study, it can be inferred that PSM as masculinizing agent may provide a veritable alternative to 17α-methyltestosterone used in food fish production, thereby minimizing the influence of anabolic steroids on fish consumers, fish culturists, and on the environment.

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Chapter 5

The effect of Pawpaw (*Carica papaya*) seed meal on the hormonal profile and reproductive parameters of pre-vitellogenic *Oreochromis mossambicus*

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5.1 Abstract

Pawpaw (*Carica papaya*) seeds have been reported to have an anti-fertility effect in humans and various animal species. This study investigated the influence of pawpaw seed meal (PSM) on the reproductive performance, and the sex hormone profile of sexually immature Mozambique tilapia (*O. mossambicus*). Pawpaw seed powder were included in a commercial tilapia diet (basal diet) at an inclusion level of 0, 10 and 30 g/kg of the basal diet, respectively, and fed to immature Mozambique tilapia of 24.81±8.54g mean weight for a period of 30 and 60 days, respectively. Blood samples were collected from four fish (two males and two females) from each replicate on the days 0, 30 and 60 respectively. Enzyme-linked immuno-sorbent assay (ELISA) procedures were used to quantify the plasma levels of 17β-estradiol and 11-ketotestosterone. The inclusion of PSM did not affect the growth and survival rate of the pre-vitellogenic OM in this study. The plasma levels of 11-ketotestosterone did not differ between the treatment groups. The 17β-estradiol levels of female fish that received 30g PSM/kg BD were significantly lower than the levels reported for female tilapia that received the BD only (2.62 ± 0.4 ng/mL vs. 5.17 ± 0.61 ng/mL). The dietary inclusion of PSM had no effect on gonad weight and the gonadosomatic index (GSI) of male *O. mossambicus*. In the females, the PSM reduced the gonad weight, GSI, fecundity and egg diameter of fish that received the treatment diets. These changes were not observed on discontinuation of feeding with the diet containing PSM which indicated that the induced changes are reversible.

5.2 Introduction

Carica papaya (Linn. Caricaceae), commonly known as pawpaw, is an important food plant that is cultivated worldwide. Apart from its nutritious fruit, different parts of the plant have been used extensively for medicinal purposes. Pawpaw plants have been credited with antibacterial, antifungal, pesticidal and molluscicidal activities (Eno *et al.*, 2000; Doughari *et al*., 2007; Jaiswal & Singh, 2008). Additionally, the seeds of pawpaw have been shown to have spermicidal, anti-implantation, abortificient and antifertility effects in mammals (Adebiyi *et al.*, 2003; Manivannan *et al.*, 2004; Udoh *et al.*, 2005). Pawpaw seeds are also reported to cause a reduction in fecundity, hatchability, egg size and gonado-somatic index in fish (Hossam & Wafaa, 2011). In the studies of Lohiya *et al*. (1999) and Pathak *et al*. (2000), it was found that the endocrine disrupting effect of pawpaw seeds

were reversed after withdrawal of treatment, with most of the reproductive parameters altered, reported to return to normal levels.

Mozambique tilapia (*Oreochromis mossambicus*) is one of the important members of the tilapia family, being outperformed by the Nile tilapia (*O. niloticus*) in terms of production efficiency (Coward & Bromage, 2000). Like other members of the tilapia family, Mozambique tilapia is very prolific both in the wild and in captivity. The prolific nature of the species is considered as the greatest hindrance to profitable culture due to the fact that precocious breeding result in a large proportion of the fish in culture systems being unmarketable, due to a large proportion of the fish being too small to contribute to optimal production. To overcome the problems of stunted growth occasioned by indiscriminate spawning in mixed tilapia culture, several methods have been used to produce mono-sex tilapia culture systems, the most common amongst them is the use of hormones to manipulate the gender of treated fish (Abucay & Mair, 1997; Desprez *et al.*, 2003). Sex reversal in fish is also practiced because of the belief that a particular gender has a growth advantage over the other (Haniffa *et al.,* 2004), which in the case of tilapia, males exhibit a faster growth rate than females (Lovshina *et al.*, 1990).

Because of the disadvantages inherent in the use of hormones to manipulate the gender of fish, which include amongst others the cost of the hormones, health hazard to workers, adverse environmental effects and unfavourable public perception of the use of hormone in food fish production (Green and Teichert-Coddington, 2000; Beardmore *et al.*, 2001), scientists have investigated the potential to use less harmful phytochemicals of plant origin like pawpaw seed meal to manipulate the gender in fish (Ampofo-Yeboah, 2013). The potential of pawpaw seeds to induce sex reversal in fish can be attributed to the presence of phytochemicals present in the seed of the plant. The chemical composition of the pawpaw seeds include fatty acids, crude proteins, crude fiber, papaya oil, carpaine, benzyl isothiocyanate, benzyglucosinate, glucotropacolin, benzylthiourea, hentriacontane, β-sitosterol, caricin, and enzyme myrosin (Krishna *et al.*, 2008). Phytoestrogens are referred to as endocrine disrupting compounds, and are capable of causing reproductive dysfunction in animals including fish (MacLatchy & Van Der Kraak, 1995; Clotfelter & Rodriguez, 2006). Some phytochemicals are believed to be estrogenic in nature, which means they either mimic the action of estrogen or they compete for estrogen receptors, thereby blocking the action of natural estrogens (Rearick *et al.*, 2014).

Ovarian development in fish is mediated by internal and also external stimuli such as temperature, photoperiod, water quality, and the presence of suitable sexual partners. On receipt of favourable internal and external stimuli, the brain (hypothalamus) transmits neural impulses which stimulate the endocrine hypothalamo-pituitary-gonadal pathways (HPG) to produce the gonadotropic hormones. The gonadotrophic hormones, follicle

stimulating hormone (FSH) and luteinizing hormone (LH) which are important products of the HPG axis, play a vital role in steroidogenesis and gametogenesis in animals including fish. Follicle stimulating hormone stimulates early stages of spermatogenesis and oocyte development in males and females respectively, while LH stimulates both gametogenesis and steroidogenesis. Follicle stimulating hormone and LH binds to their receptors in the gonads, and stimulate the synthesis and secretion of the sex steroids, estradiol 17β and androgens. Estradiol 17β is responsible for the development of oogonia and vitellogenesis in females, and androgen is responsible for spermatogenesis in males. After the vitellogenic phase, oocytes may remain dormant until favourable environmental conditions stimulate the final maturation of the oocytes, where after ovulation is initiated by 17α, 20β dihydroxy-4-pregnen-3-one (DHP), a maturation inducing steroid (MIS) (Yaron & Levavi-Sivan, 2011). The 11-ketotestosterone produced early during testicular development, is the main androgen in fish and is an important mediators of masculinization, while DHP is also responsible for initiation of maturation of spermatozoa in males (Blasco *et al.*, 2012).

The gonadal concentration of androgen relative to estrogen during sexual differentiation determines the sex of the individual, and the ratio of these two steroids are controlled by the aromatase enzyme (Bogart, 1987). In the brain and gonadal tissues, the enzyme cytochrome P450 aromatase converts androgen to estrogen, thereby modulating the concentration of estrogen in the gonadal tissues and in the blood circulation (Ankley *et al.*, 2002). Scientists believe that the rate of secretion of hormones from the pituitary and gonads and the rate of clearance determines the eventual concentration in the plasma (Cornish, 1998).

Some exogenous compounds have the potential to disrupt the normal synthesis and signalling of the endogenous hormones. These exogenous compounds that interferes with the endogenous hormone reception is termed endocrine disrupting compounds (Casanova-Nakayama *et al.*, 2011). Changes in reproductive parameters such as fecundity, gonado-somatic index and sex steroid hormones; 17β - estradiol and 11-ketotestosterone concentration in plasma can be used as an endpoint indicators of endocrine disrupting chemicals in fish (Ankley *et al.*, 2002; Dang *et al.*, 2011).

As there is little information available on the influence of pawpaw seeds on the reproductive endocrinology of *O. mossambicus*, the present study was aimed at evaluating the effect of the phytochemicals contained in the seeds of the plant on the reproductive hormone profile and other reproductive parameters of the fish in greater depth.

5.3 Materials and Methods

5.3.1 Experimental location and facilities

The experiment was conducted in a one tier plastic water re-circulatory system built in a glass house at the Aquaculture unit of the Welgevallen experimental farm of the University of Stellenbosch. The plastic tanks have a dimension of 70x40x38cm (LxWxH) and volume of 90 L. It also has aeration, filtration and water heating facilities incorporated into its design.

The physicochemical parameters of the culture water were monitored daily. The mean water temperature recorded was $26.030.61^0$C, dissolved oxygen was 6.77 ± 0.78 mg/l while pH and conductivity were 6.42 ± 0.18 and 210 ± 8.02 respectively, please refer to Chapter 3 for more details.

5.3.2 Experimental animals and husbandry

A total of 1 000 juvenile *O. mossambicus*, obtained from the Rivendell Hatchery, Grahamstown, South Africa were used in the study. The fish were stocked in a holding facility upon arrival at the Welgevallen Experimental Farm where they were acclimatized for three weeks. During the acclimatization period they were fed *ad libitum* twice daily with a standard (basal) tilapia diet.

The stocking density was 50 fish per replicate and the fish were fed *ad libitum* three times a day (9.00 – 9.30 h, 13.00 – 13.30 h and 17.00 – 17.30 h) with the experimental diets. The waste and uneaten food in the aquaria were carefully removed daily by siphoning and the tanks refilled with fresh water. Dead fish were removed from culture tanks immediately there was mortality.

5.3.3 Experimental diets

Fresh seeds were obtained from large quantities of ripe pawpaw obtained from fruit vendors in Stellenbosch, Western Cape, and dried in-doors. The dried seeds were blended to a fine powder using a laboratory grinder (Knifeter 1095, FOSS), and stored in Ziploc bags for later use. The standard (basal) diet consisted of a commercial tilapia diet (40% crude protein, Aqua-Nutro, Nutroscience (Pty) Ltd, Malmesbury, South Africa). The pawpaw seed meal (PSM) was added to the basal diets according to the inclusion level for that treatment group. A measured quantity of the basal diet and the experimental *C. papaya* seed powder were mixed thoroughly in Macadams baking system (model: SM-401). To enable pelleting of the feed, lukewarm water (200 mL/kg of feed) was added to the mixture during mixing. The mixture was pelleted in an extruder and oven-dried in a CFW Envirowatch 5 (model: Ø560) oven, and then stored in airtight containers till later use.

5.3.4 Experimental design

The experimental set up based on the inclusion levels and duration of the feeding period of the experimental diet was as shown in Table 5.1. There were five experimental treatment groups with four replicates each.

Table 5.1 Inclusion level of pawpaw seed meal (PSM) and designation of treatment groups.

Treatment	Inclusion level of PSM	Designation
Basal diet (BD; negative control) for 2 months	No inclusion	BDM2
BD + PSM for 1 month	10 g PSM/ kg BD	P10M1
	30 g PSM/ kg BD	P30M1
BD + PSM for 2 months	10 g PSM/ kg BD	P10M2
	30 g PSM/ kg BD	P30M2

5.3.5 Data recorded

Measurement of body weight and length

On the day of stocking, the entire fish in the whole treatments and replicates were weighed individually and their total and standard lengths measured. Taking the weight, total and standard lengths of the entire experimental fish were repeated on the 30th and 60th days of the study. The total and standard lengths of the specimen were measured using a measuring board graduated in centimeters. The total length was measured from the anterior most extremity of the fish to the end of the tail fin to the nearest centimeters. The standard length was measured from the most anterior extremity to the base of the tail fin. The base was identified by a crease when the tail was bent sharply from side to side. Top loading balance (Electronic Balance, UWE, HGS-300, capacity: 300 x 0.01g, Serial # P9440) was used to measure the body weight of the fish samples to the nearest grams.

Measurement of reproductive parameters.

At the end of 60 days experimental period, four fish from each replicate (two males and two females) were randomly selected and after taking their weights, dissected to ascertain the maturation of the gonads, fecundity, and egg diameter and the gonado-somatic index calculated.

The diameter of 12 eggs randomly taken from the anterior, middle and posterior parts of the ovary was measured using a binocular microscope. The long and short axis of each egg were measured and the mean taken as the diameter of the egg (Abdelhak *et al.* 2013).

The specific growth rate was calculated using the formula:

SGR = $\dfrac{LnW_f - LnW_i}{T\ (days)}$ X100   (Tran-duy et al. 2008)

Where W_f = final weight and W_i = initial weight

Dissected ovaries were preserved in 10% formalin for 3 weeks, later they were gently agitated to separate the eggs from the ovarian tissues and then the formalin decanted out. The eggs were washed by adding clean water in a beaker containing the eggs, after gentle agitation, the water was filtered out. Entire eggs were put in a clean filter paper and weighed, a sub-sample of the eggs were weighed then counted.

The fecundity of each female fish sampled was determined using the formula:

Fecundity = $\dfrac{\text{Total weight of ovary}}{\text{Weight of sub sample}}$ X number of mature eggs in sub sample.

(Gaikwad *et al.*, 2009)

The gonado-somatic index (GSI) was determined using the formula:

GSI = $\dfrac{\text{Gonad weight (g)}}{\text{Body weight (g)}}$ X100

Determination of steroid hormone levels

Blood samples were collected from four fish (two males and two females) from each replicate on Day 0 of the experiment from the caudal circulation with the aid of heparinized 3mL disposable plastic syringes and a 21 gauge disposable hypodermic needle. Blood samples were again collected Day 30 and Day 60 of the experiment. The blood samples were transported to the laboratory immediately after collection, and processed to collect the plasma fraction. Samples were centrifuged at 3500 rpm for 10 minutes at 4 °C using Eppendorf centrifuge (Model 5804R), and stored at -20°C until analysed. The reproductive hormones, 17β - estradiol and 11 keto- testosterone were quantified using ELISA kits specific for the quantitation of fish hormones. The 11-ketotestosterone hormone was assayed using fish specific 11-ketotestosterone EIA kit (Item №: 582751; Batch: 0468795) manufactured by Cayman Chemical, USA while 17β - estradiol was assayed using Fish Estradiol (E2) ELISA kit (Catalog №: CSB-E13017fh; Lot: C2489421809) manufactured by CUSABIO BIOTECH Co, China. The procedures for the assays were according to manufacturer's instruction and were done in duplicate. For details refer appendices.

5.3.6 Statistical analysis

The results are presented as means ± SE. The data were analysed by one way analysis of variance (ANOVA), with confidence interval of 95%. Variant means were separated by using Bonferroni (Dunn) t test.

5.4 Results

5.4.1 Morphometric parameters

Results of the morphometric parameters measured on the 60th day of the feeding trial indicated no significant difference in any of the parameters. The 60th day sampling recorded maximum total length (13.93±0.19cm) and minimum (13.64±0.21cm) obtained in treatment P10M1 and P30M2 respectively. The maximum mean standard lengths of the treatment groups at the end of the experiment (11.21±0.17cm) was recorded for P10M2 group while the minimum (10.71±0.13cm) was recorded for P30M2 group. The maximum (48.36±1.58g) and minimum (44.24±1.82g) weights were obtained in groups P10M2 and P30M2 respectively [Table 5.2]. Furthermore, the specific growth rate (SGR) ranged between 0.96±0.08 and 1.11±0.09 obtained in the treatment group BDM2 and P10M2 respectively. The SGR also indicated no significant difference between different groups. There were high survival rate in all the treatment groups ranging between 96.5 – 97.5% (Table 5.2).

Table 5.2 Morphometric parameters (mean±SE), specific growth rate and survival rate of 2 months old *O. mossambicus* of mean weight 24.81±8.54 g that received diets containing pawpaw seed meal for 30 and 60 days, respectively.

Treatment	Total length (cm)	Standard length (cm)	Weight (g)	Specific growth rate	Survival (%)
BDM2 (Control)	13.75±0.17	10.8±0.18	44.56±1.72	0.96±0.08	97.5
Period 30 days					
P10M1	13.82±0.13	10.86±0.24	46.25±1.70	1.04±0.07	96.5
P30M1	13.65±0.22	11.1±0.14	45.7±1.62	1.03±0.10	96.5
Period 60 days					
P10M2	13.93±0.19	11.21 ± 0.17	48.36±1.58	1.11±0.09	97
P30M2	13.64±0.21	10.71±0.13	44.24±1.82	1.0±0.07	96.5

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5.4.2 The influence of PSM on 17β-estradiol levels

At the beginning of the experiment, no significant difference in the plasma concentration of 17β-estradiol (E2) as determined for females, was observed (Figure 5.1) however the P30M2 group had the maximum (3.44±0.82 ng/mL) while the P10M1 group with 2.43±0.75 ng/mL recorded the minimum concentration of E2. By the 30th day post exposure P30M2 group that were fed 30 g/kg of PSM had their E2 concentration declined drastically from the highest of all the treatment group on the day 0 to lower than the rest except P30M1 group which also received 30 g/kg of the experimental diet [Figure 5.5]. The maximum concentration of E2 at day 30 (4.89±0.72 ng/mL) were recorded for the control group that were fed only BD while the minimum (2.1±0.82 ng/mL) was recorded for P30M1 group, however these differences were not statistically significant.
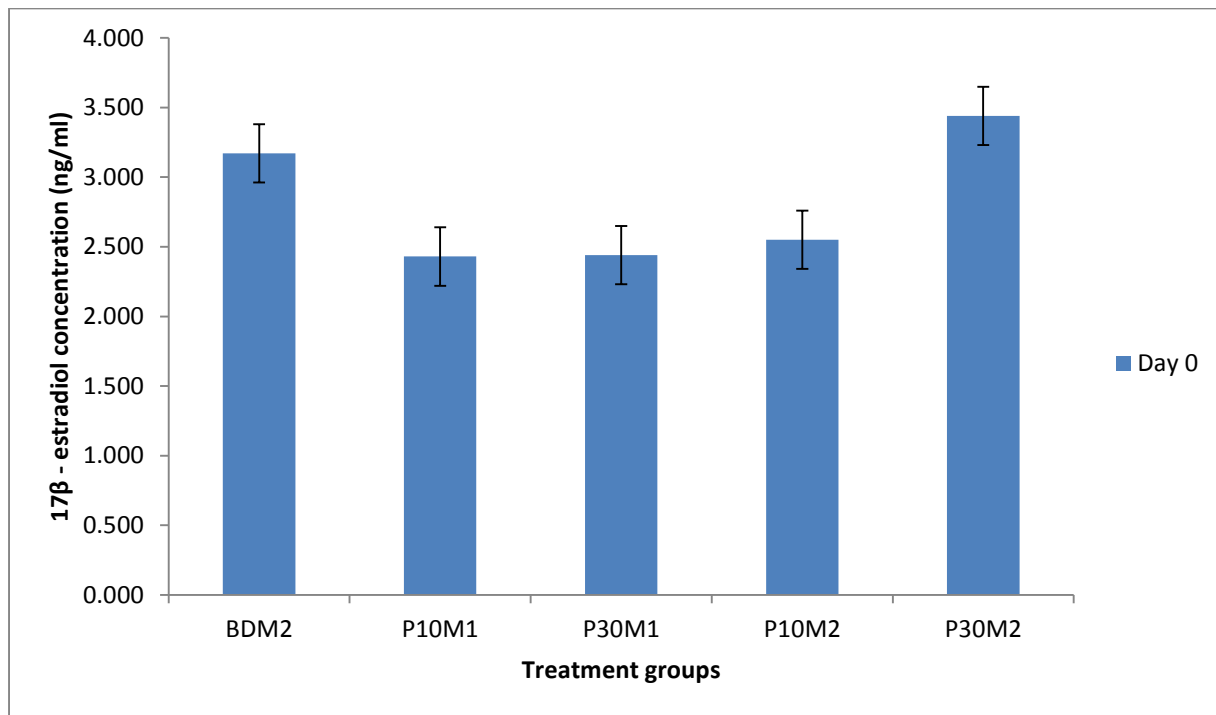
Figure 5.1 17β - estradiol Concentration (ng/mL) in female *O. mossambicus* fed graded levels of pawpaw seed meal on Day 0.
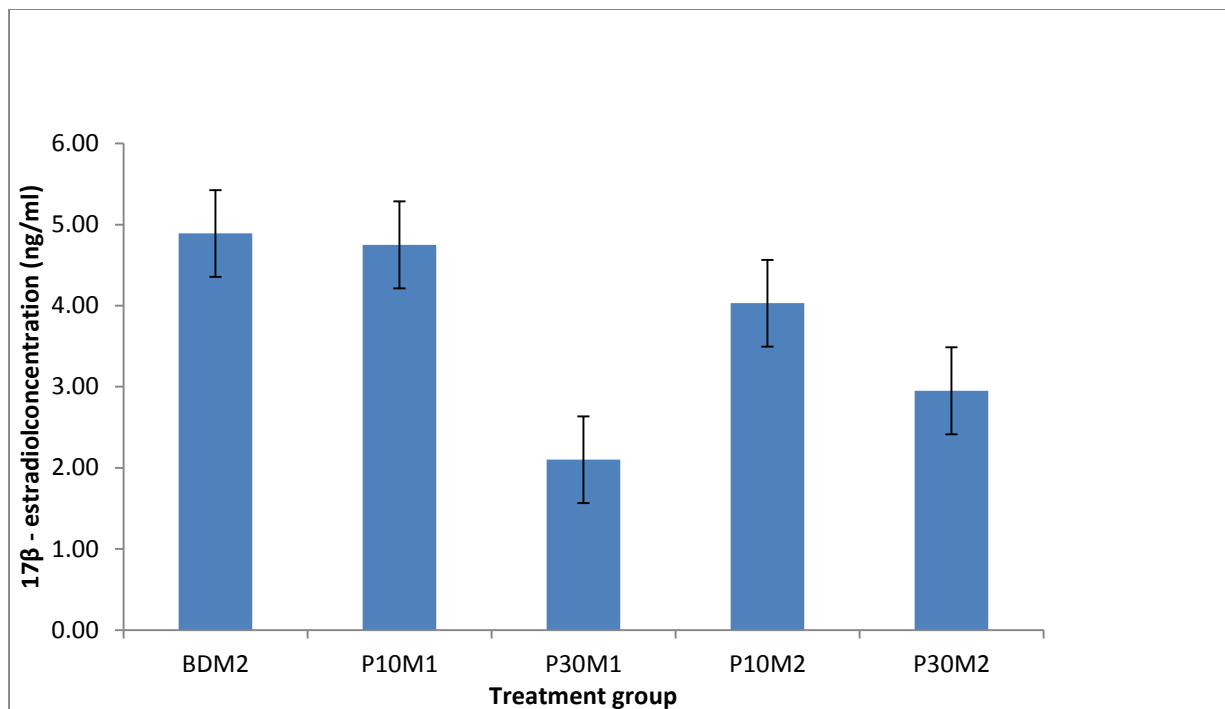
Figure 5.2 17β - estradiol Concentration (ng/mL) in female *O. mossambicus* fed graded levels of pawpaw seed meal at day 30.

The decline in the plasma concentration of E2 among the P30M2 group that received 30 g of PSM/kg of BD for the whole experimental period continued on the 60th day. However the depression in the 17β-estradiol level in P30M1 group recovered after the feeding with 30 g/kg PSM diet was discontinued beginning from the 31st day of the trial [Figure 5.3]. The lowest concentration of E2 (2.62±0.4 ng/mL) recorded for P30M2 group that were fed 30 g/ PSM/kg of BD was significantly lower (P < 0.05) than 5.17±0.61 ng/mL obtained among the control group (BDM2) as shown in Table 5.3. Among the males, the trend of E2 concentration in the plasma was not well defined. At the end of the experimental period, the highest concentration (2.72±0.59 ng/mL) was obtained in P10M2 group which received 10 g/kg of the PSM for 60 days while the least was in group P30M2 (1.9±0.52 ng/mL) though the difference was not significant (P > 0.05) statistically.
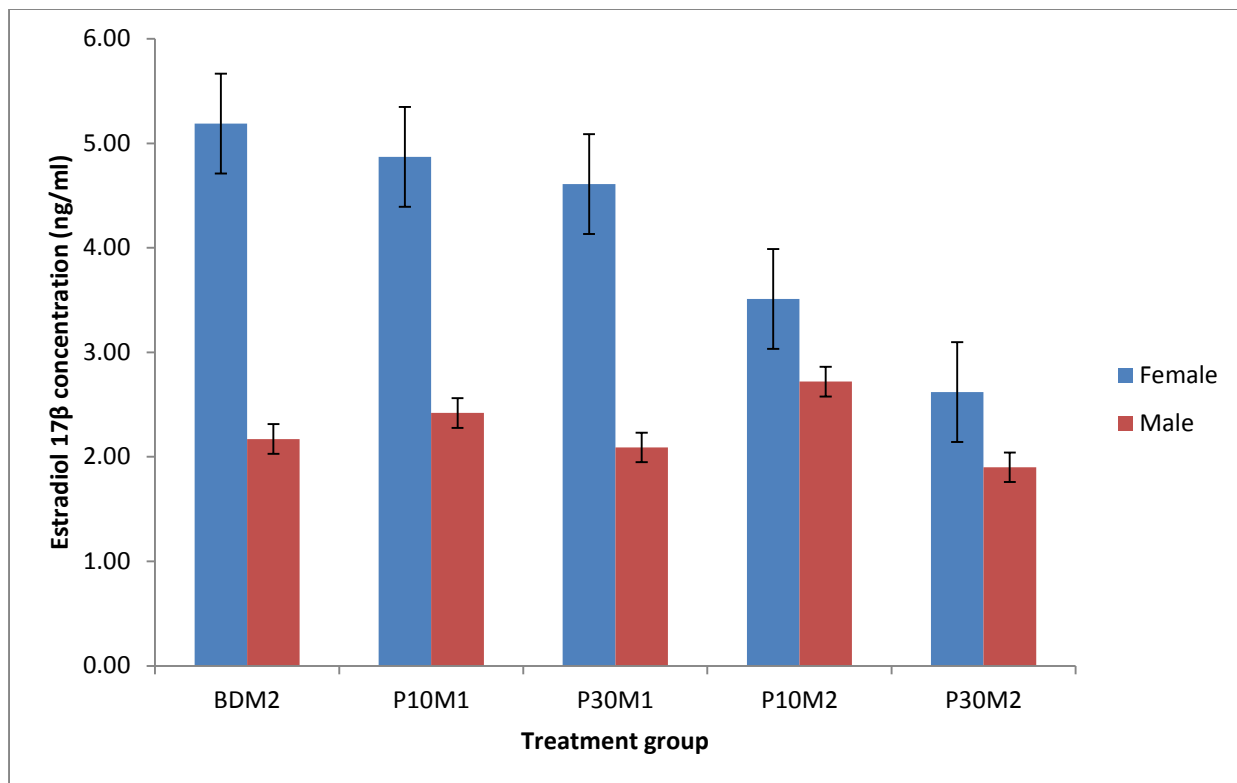
Figure 5.3 17β - estradiol Concentration in male and female *O. mossambicus* fed graded levels of pawpaw seed meal at day 60.

Table 5.3 17β-estradiol plasma levels (mean±SE) of *O. mossambicus* females that received a basal diet supplemented with pawpaw seed meal for 30 days, and 60 days, respectively.

Treatment	Day 0	Day 30	Day 60
BDM2 (Control)	3.17±1.08	4.89±0.81	5.17±0.61[a]
Period 30 days			
P10M1	2.43±0.53	4.75±1.11	4.87±0.81[ab]
P30M1	2.44±0.24	2.1±0.45	4.61±0.85[ab]
Period 60 days			
P10M2	2.55±0.31	4.03±0.86	3.51±0.58[ab]
P30M2	3.44±0.92	2.95±0.57	2.62±0.4[b]

Columns with different superscripts differ significantly (P < 0.05)

5.4.3 Influence of pawpaw seed meal on serum 11-Ketotestosterone levels

There were no definite trends established in 11-ketotestosterone (11-KT) concentration in both male and female *O. mossambicus* in this study that can be attributed to the experimental diet. Among the males, the P30M1 group

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had the maximum concentration of 11-KT (2.74±0.76 ng/mL) while BDM2 group had the minimum (0.55±0.76 ng/mL) at the start of the feeding trial though the difference was not statistically significant (P > 0.05).

Table 5.4 Mean ($\pm SE$) Plasma concentration of 11-Ketotestosterone (ng/mL) in male and female *O. mossambicus* fed graded levels of pawpaw seed meal.

Treatment	Day 0		Day 30		Day 60	
	Male	Female	Male	Female	Male	Female
BDM2 (Control)	0.55±0.76	1.41±0.85	4.11±0.81	4.27±0.88	0.68±0.16[a,b]	0.23±0.18
Period 30 days						
P10M1	1.16±0.7	2.29±0.76	2.43±0.81	5.0±1.00	0.49±0.16[b]	0.22±0.18
P30M1	2.74±0.76	1.34±0.76	1.23±0.75	2.04±0.88	0.48±0.15[b]	0.27±0.17
Period 60 days						
P10M2	0.79±0.85	1.84±0.85	2.35±0.88	2.09±0.81	1.31±0.16[a]	0.32±0.18
P30M2	1.39±0.65	0.61±0.7	2.25±0.81	3.47±0.99	0.91±0.16[a,b]	0.21±0.15

[a, b]Columns with different superscripts differ significantly (P < 0.05)

Also there was no significant difference in the concentration of 11-KT between the different treatment groups on the 30th and 60th day post exposure [Table 5.4]. The same pattern recorded for the males were also obtained among the females with no significant difference (p>0.05) in the plasma level of 11-KT during the three sampling days [Table 5.4].

5.4.4 Influence of pawpaw seed meal on reproductive parameters

Data for mean gonad weight, gonadosomatic index (GSI), absolute fecundity, relative fecundity and egg diameter from *O. mossambicus* exposed to different inclusion levels of pawpaw seed meal are presented in Table 5.5. There were no significant differences (P > 0.05) in the mean gonad weight and GSI of males between the different treatment groups. The heaviest gonad weight (0.47±0.1 g) in males was recorded for the control group (BDM2) while the minimum (0.41±0.1 g) was in group P10M2. Also the maximum mean GSI (0.5±0.2) was recorded for BDM2 group while the least (0.4±0.2) in P30M2 group. In the females however the control group had a significantly (P < 0.05) higher gonad weight when compared with those of group P30M2 that were fed 30 g/kg of PSM for 60 days, P30M1 group fed 30 g/kg for the first 30 days and P10M2 that were fed 10 g/kg for 60 days. The control group also recorded a significantly (P < 0.05) higher GSI, absolute fecundity, relative fecundity and egg diameter compared to the treatment groups P30M2, P30M1 and P10M2 [Table 5.5].

Table 5.5 Mean($\pm SE$) Reproductive parameters of *O. mossambicus* of different treatment groups fed graded levels of pawpaw seed meal.

Treatment	Gonad weight (g)		Gonadosomatic index		Absolute Fecundity	Relative Fecundity	Egg Diameter
	Male	Female	Male	Female			
BDM2 (Control) Period 30 days	0.47±0.1	2.41ᵃ ±0.1	0.5±0.2	4.95ᵃ ±0.2	164.6ᵃ±9.2	3.4ᵃ ±0.1	1.56ᵃ ±0.1
P10M1	0.45±0.1	2.15ᵃ,ᵇ±0.1	0.49±0.2	4.62ᵃ,ᵇ±0.2	162.4ᵃ±9.7	3.5ᵃ±0.1	1.53ᵃ,ᵇ±0.1
P30M1	0.42±0.2	1.63ᵇ±0.1	0.42±0.2	3.63ᵇ,ᶜ±0.1	110.3ᵇ±8.6	2.4ᵇ±0.1	1.19ᵇ,ᶜ±0.2
Period 60 days							
P10M2	0.41±0.2	1.72ᵇ±0.1	0.43±0.2	3.42ᵇ,ᶜ±0.1	118ᵇ±9.4	2.6ᵇ±0.2	1.2ᵇ,ᶜ±0.2
P30M2	0.42±0.1	1.58ᵇ±0.1	0.4±0.2	3.34ᶜ±0.2	99.1ᵇ±9.2	2.2ᵇ±0.1	1.16ᶜ±0.1

[a, b, c] Columns with different superscripts differ significantly (P < 0.05)

5.5 Discussion

The morphometric characteristics of the control and pawpaw seed meal (PSM) exposed *O. mossambicus* obtained from the study indicated that there was no significant difference (P > 0.05) in the total length, standard length and weight among all the treatment groups from the beginning of the experiment to its termination. A comparison of the mean weight of the different treatment groups obtained for the entire experimental period as shown in table 5.2 indicates a uniform increases in the weight of the treated fish and the control group. It can be deduced from the result of the study that PSM has no effect on the morphometric parameters of *O. mossambicus* juveniles at an inclusion level of 30 g/kg of basal diet. The specific growth rate (SGR) obtained in the study had the minimum (0.96±0.08) and maximum (1.11±0.09) values obtained in the control group (BDM2) and the group that were fed 10 g of PSM/kg of BD for 60 days (P10M2) respectively. The SGR did not differ between the different treatment groups. There were high survival rate in all the treatment groups with the minimum and maximum values of 96.5 and 97.5 respectively. The fact that the control group had no growth advantage or survival rate different from the pawpaw seed meal treated group shows that the diet has no impact on the growth and survival rate up to inclusion level of 30 g/kg. This result is consistent with earlier findings of Ampofo-Yeboah (2013) who reported no significant difference in the total length, weight and body depth of *O.*

mossambicus fed pawpaw see meal. Ekanem & Okoronkwo (2003) reported decrease in weight of *O. niloticus* fed 9.8 g of PSM/kg of standard tilapia diet but the difference was not significant. The insignificant differences in the weight and other morphometric parameters observed in this study was also in agreement with previous studies conducted on the use of pawpaw seed extracts as reproductive inhibitors in laboratory animals such as albino rats (Lohiya *et al.*, 1994), they concluded that the sterility induced in male rats are dose and duration dependent and has no effect on its body weight.

The availability of ELISA and other immunoassays for the quantitative determination fish reproductive hormones (Ishikawa *et al.*, 1991; Porstmann & Kiessig, 1992; Cuisset *et al.*, 1994; Nash *et al.*, 2000) has made possible investigations of the variation in plasma sex steroid levels in relation to season (Cornish, 1998), pollutants (Hansen *et al.*, 1998; Hintemann *et al.*, 2006) and response to xenobiotic treatments (Arukwe *et al.*, 1999). There are three distinct phases in the reproductive cycle of both male and female *O. mossambicus*, they include breeding season, resting and gonadal recrudescent seasons and the plasma concentration of steroid hormones are seasonal dependent (Cornish, 1998). The rate of secretion of hormone from the gland and gonad and its rate of clearance determines its concentration in the plasma. During vitellogenesis an increase in plasma levels of estrogen mainly 17β-estradiol has been found to correlate with the growth of vitellogenic oocytes (Yaron & Levavi-Sivan, 2011). At the start of the experiment, the levels of the sex steroids measured at day zero were similar for all the treatment groups. In the females however, after the 30 day feeding with the PSM the sex steroids in treated groups were lower than the control group. The maximum concentration of 17β-estradiol at day 30 (4.89±0.72 ng/ml) were recorded for the control group that were fed basal diet only while the minimum (2.1±0.45 ng/mL and 2.95±0.57 ng/mL) were obtained in P30M1 and P30M2 groups respectively and both were fed 30 g PSM/kg of BD for the first 30 days, however these differences were not statistically significant.

Even though there was no significant difference in 17β-estradiol concentration at this stage, the P30M1 and P30M2 values positively correlated with the control with R^2 value of 0.413. The decrease in the plasma concentration of 17β-estradiol among the P30M2 group that received 30 g/kg pawpaw seed meal for the whole experimental period continued on the 60th day. However the depression in the 17β-estradiol level in P30M1 group recovered after the feeding with 30 g/kg PSM diet was discontinued beginning from the 31st day of the trial [Figure 5.3]. The fact that 17β-estradiol concentration returned to normal level after the cessation of treatment in group P30M1 indicates reversibility of the effect of the PSM on *O. mossambicus* up to inclusion level of 30 g/kg of basal diet. This is in agreement with the reports of other workers who reported induction of reversible sterility in laboratory rats by the use of PSM (Lohiya *et al.*, 1994; Pathak *et al.*, 2000). The lowest concentration of 17β-estradiol (2.62±0.4 ng/mL) recorded for P30M2 group that were fed 30 g/kg PSM at day 60

was significantly lower (P < 0.05) than 5.17±0.61 ng/mL obtained among the control group (BDM2) as shown in Table 5.4. This result is in agreement with the earlier work of Abdelhak *et al.* (2013) who fed *O. niloticus* PSM ranging from 60,90, and 120 g/kg of basal diet and recorded significantly lowered 17β-estradiol in all the treated group compared with the control (zero PSM diet). The fact that pawpaw seed meal resulted in lower 17β-estradiol levels in females may explain the sex reversal of genetic females to phenotypic males of *O. mossambicus* attributed to the seed meal (Ampofo-Yeboah, 2013), and may also account for the inhibition of reproductive activities reported by Hossam and Wafaa (2011) and Abdelhak *et al*. (2013). Ampofo-Yeboah (2013) included 15 g PSM/kg of basal diet of *O. mossambicus* and recorded 65% masculinization.

In the brain and gonadal tissues, an enzyme cytochrome P450 aromatase converts androgen to estrogen thereby modulating the concentration of estrogens in the gonadal tissues and in the blood circulation (Ankley *et al.*, 2002). It has been suggested that phytoestrogens exhibit an estrogenic or anti-estrogenic effect in the presence of endogenous estrogens, i.e. they can mimic the effect of estrogen or block the function of estrogen (Clotfelter & Rodriguez, 2006; El-Sayed *et al*., 2012). This inhibition of the function of the endogenous estrogen may explain the mode of action of PSM in depressing the plasma levels of estrogens in female *O. mossambicus* reported in this study. However from available literature it is yet to be determined whether the phytoestrogens accomplish this by inhibiting the action of the P450 aromatase enzyme. Jaiswal and Singh (2008) were of the opinion that the efficacy and /or toxicity of the pawpaw seed depends on the dose and duration of the application. There are however, contradictory reports on the appropriate dosage of the phytochemical needed. Hossam & Wafaa (2011) used 3 g/kg (low dose) and 6 g/kg (high dose) of PSM in their study, and achieved reversible and permanent sterility, respectively, in the Nile tilapia (*O. niloticus*). Abdelhak *et al*. (2013) included PSM meal at 60 g, 90 g and 120 g/kg of a commercial diet, and found that 60 g/kg as a low dose resulted in reversible sterility, whereas an inclusion level of 120 g/kg as a high dose resulted in permanent sterility.

The concentrations of 17β-estradiol [E2] in males were significantly lower than in the females as illustrated in figure 5.4, however among the males of different treatment groups; the trend of E2 concentration in the plasma was not well defined. At the end of the experimental period, the highest concentration (2.72±0.59 ng/mL) was obtained in P10M2 group which received 10 g/kg of the pawpaw seed meal for 60 days while the least (1.9±0.52 ng/mL) was in group P30M2 that were fed 30 g/kg for 60 days, though the difference was not significant (P > 0.05) statistically. The fact that the males had significantly lower 17β-estradiol was expected since the hormone play major role in oogonia proliferation and final oocytes maturation in female fish (Yaron & Levavi-Sivan, 2011).

Testosterone and 11-ketotestosterone are the main androgens found in fish, however 11-ketotestosterone is always quantitatively more than testosterone (Rinchard *et al.*, 2002; Ribeiro *et al.*, 2012). Cholesterol is the precursor of testosterone in response to gonadotropin hormone stimulation (Peng *et al.*, 2015). In teleost, testosterone is converted to a more potent androgen, 11-ketotestosterone by the hydroxylation enzyme P450 dehydrogenase (Baroiller *et al.*, 1999; Yaron & Levavi-Sivan, 2011). In terms of masculinization, 11-ketotestosterone is considered as one of the main mediators of masculinization and is produced early during testicular development *(*Blasco *et al.*, 2012). In this study, there were no definite trends established in 11-ketotestosterone concentration in both the male and female *O. mossambicus* that can be attributed to the experimental diet. Also there was no significant difference observed when concentrations among different groups were compared. This is contradictory to the report of Abdelhak *et al*. (2013) who reported a significant decrease in the level of testosterone in *O. niloticus* fed various levels of PSM although their inclusion level were much higher (60, 90 and 120 g/kg of basal diet) than in the present study. The study of Abdelhak *et al.*, (2013) was the only study in fish that reported that pawpaw seed meal significantly decreased the level of testosterone or 11-ketotestosterone. In laboratory animal research, Pathak *et al.* (2000) reported no effect on testosterone levels but mild estrogenicity in rats treated with a chloroform extract of pawpaw seeds. Lohiya *et al.* (2005) also reported no effect of pawpaw seed extract on the serum testosterone of albino rats.

Gonad weight and gonadosomatic index (GSI) are indicators of gonadal maturation in fish. The GSI represents the relationship between the gonad weight and body weight, and is more suitable than absolute gonad weight as an indicator of maturity in male fish (Höerstgen-Schwark & Langholz, 1998). In females, apart from gonad weight and GSI, the visual assessment of the stages of the gonadal development is equally useful in determining the maturity status of the gonad (Armstrong & Witthames, 2012). The GSI of male *O. mossambicus* varies between 0.35 to 0.92% (Shubha & Reddy, 2011). According to Bhatta *et al.*, (2012) male *O. mossambicus* usually have their highest GSI of up to 1% of the body weight during the spawning season. In the males, there was no significant difference in the end point indicators of reproductive impairment, GSI and gonad weight recorded in this study, however the control group had GSI of 0.5% while the group that were fed 30 g/kg of pawpaw seed meal for 60 days recorded 0.4%. This observation seemed to agree with the findings of Abdelhak *et al*. (2013) who found no significant difference in the GSI of *O. niloticus* even at an inclusion level of 120g of PSM/kg of basal diet. Ampofo-Yeboah (2013) recorded no influence of PSM on the GSI of male *O. mossambicus*. However in a related study, Sadekarpawar & Parikh (2013) reported a significant reduction in the GSI of *O. mossambicus* male treated with a sub-lethal concentration of Librel™, an EDTA chelated micronutrient mixture. However, in the laboratory animal research, there are conflicting reports on the effect of pawpaw seed on gonad weights. Lohiya *et al.* (1994) reported a reduced testicular weight in male rats while Pathak *et al.* (2000) reported no effect of

the pawpaw seed on the weight of testis but recorded decreased sperm count, motility and viability of the spermatozoa.

In the females, the end point indicators of reproductive impairment such as gonad weight and GSI were all significantly reduced in the pawpaw seed treated groups [Table 5.6]. This is also in agreement with the work of Abdelhak *et al.* (2013), who recorded significant reduction in GSI of *O. niloticus* fed diet containing PSM. Fish fecundity is the rate of egg production by female fish in a given period (breeding season). Fecundity and survival rate are the main factors that determine the population size of fish (Campos-Mendoza *et al.*, 2004). In this study there was significant ($P < 0.05$) reduction in fecundity and oocytes diameter in the pawpaw seed treated group compared with the control as shown in table 5.6. The control group had a mean fecundity of 164.6$\pm$9.2 eggs while the group that was fed 30 g/kg of PSM has mean fecundity of 99.1$\pm$ 9.2 eggs. In a related study, Jegede (2010) reported a significant decrease in GSI and fecundity in *O. niloticus* fed *Hibiscus rosa sinensis* leaf meal, whereas Mlambo *et al.* (2009) reported no significant difference in the GSI of male and female *O. mossambicus* exposed to the persistent pesticide DDT. There are conflicting reports on the actual fecundity of *O. mossambicus,* Mohamed *et al*. (2013) reported that fecundity of *O. mossambicus* range between 488 and 1368, while (Coward & Bromage, 2000) stated that the fecundity of *O. mossambicus* can be less than 350 eggs. Blay (1981) reported on the fecundity of a related species, *Sarotherodon galilaeus*, to vary between 69 and 302, with a mean of 149 eggs. The fecundity recorded in this study was low, 99.1$\pm$ 9.2 to 164.6$\pm$9.2 eggs and the reason for this could be that the culture environment (glass aquaria) may not be the ideal for optimum growth and sexual development. Also the temperature (24.30 – 26.70^{0}C) of the culture water recorded during the experimental period was suboptimal which probably affected the gonadal development since according to (Azaza *et al*., 2008) the optimum temperature for tilapia culture is 26 – 30°C.

During the last sampling, fry were observed in all the tanks that housed the control fish and some of the tanks which contained the groups that were fed 10 g/kg PSM for the first 30 days and continued with the basal diet whereas, none was observed in the tanks which housed the groups fed 30 g/kg and those that received 10 g/kg for the whole experimental period of 60 days. The explanation for this observation is that the PSM may have affected the gonadal maturation and/or reproductive potential of the treated fish to the extent that there were no reproductive activities taking place. The fact that those fed 10 g of PSM /kg of BD for the first 30 day recorded breeding activities while their counterparts fed the same 10 g of PSM for the whole 60 days did not breed proves that they may have recovered from reproductive inhibition after withdrawal of treatment. This observation was in agreement with the reports of earlier workers that pawpaw seed induced reproductive inhibitions are reversible (Pathak *et al.*, 2000; Hossam & Wafaa, 2011; Abdelhak *et al*., 2013).

5.6 Conclusions

It was found in this study that the levels of the female 17β-estradiol hormone were depressed by the addition of PSM in the diet of *O. mossambicus* while 11-ketotestosterone was not affected. It also inhibited reproductive activities in treated groups without an untoward effect on growth and survival of the fish. It is clear from the results presented in this study that PSM are suitable for use in *O. mossambicus* culture as a source of control for indiscriminate spawning and overcrowding for juveniles fish, up to a concentration of 30 g/kg. From the available literature, it could be concluded that the findings from this study are the first report of effect of PSM on the reproductive hormone profile of *O. mossambicus*, as well as the impairment of gondala function when PSM are included at levels of 20g/kg BD and higher.

Future studies need to focus on the effect of the PSM on hatchability of eggs spawned and also on sperm quality to determine whether the viability and fertilizing capacity of the germ cells are compromised. Presence of fry in the culture tanks of the control and low dose group and none in those of high dose group suggests that future studies should focus on reproductive trials with fish receiving low and high doses to determine whether the reproductive ability of the treated fish will contribute to overcrowding of pond systems. There is also a need to determine the influence of PSM on the role of the liver in the production of vitellogenin in both male and female *O. mossambicus*, for the absence of vitellogenin in females indicates reproductive impairment while its abnormal presence in males (vitellogenin induction) is an indication of endocrine disruption.

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Chapter 6

Effect of Pawpaw (*Carica papaya*) seed meal on the liver, haematological and biochemical parameters of juvenile *Oreochromis mossambicus*

6.1 Abstract

Pawpaw seed meal (PSM) can be used as an antifertility agent in animals, however, there is no information on its potential toxicological effect on Mozambique tilapia (*O. mossambicus*). In this study, the effect of PSM on the liver, blood haematological and biochemical parameters of *O. mossambicus* juveniles was assessed to ascertain its suitability as a reproductive suppressant. The PSM was administered at an inclusion level of 10g and 30 g/kg of a commercial tilapia diet (basal diet, BD) for 30 and 60 days, respectively. The potential toxicological effect was assessed by determining the extent of change in the normal haematological (red blood cell count, haematocrit, haemoglobin, MCV, MCH, MCHC, thrombocyte count, absolute white blood cell and differential cell counts) and biochemical (cholesterol, total protein, albumin, globulin and glucose) parameters. Haematological and biochemical profiles of different treatment groups did not differ throughout the course of the investigation. Liver weight and hepatosomatic index values of the treated fish were comparable to those of the control. Histological observation showed a minimal degree of alteration in the architecture of the liver, with degeneration and vacuolization of hepatocytes observed in less than 10% of the members in the group that received a diet containing 30 g PSM/kg BD for 60 days. No changes in liver architecture was observed in the groups that received a diet containing 30 g PSM/kg BD for 30 days, suggesting a possible reversibility/recovery of the liver after cessation of PSM intake. These findings suggest that PSM do not have any side effects that will influence the optimal function of the liver and its role in vitellogenesis. The absence of any effect of the blood parameters measured in this study also indicate that PSM has no compromising influence on the immune system of the fish, indicating that PSM can be considered as a safe alternative to induce masculinization in Mozambique tilapia, and therefore act as a reproductive inhibitor.

6.2 Introduction

Mozambique tilapia (*Oreochromis mossambicus*) is one of several tilapia species that are commonly cultured in Sub-Saharan Africa. The tilapia species, especially *O. mossambicus*, are characterized by their easy adaptability to various environmental conditions, and an exceptional tolerance to high salinity (Kamal & Mair, 2005). The ease of propagation makes tilapia one of the most preferred cultured food fish worldwide, with an annual production of over 850 metric tonnes (Coward & Bromage, 2000). Tilapia species consume a wide variety of natural food sources, exhibit a rapid growth rate and as a consequence, tilapia can attain sexual maturity in less than four months, and they spawn easily in captivity. According to (El-saidy & Gaber, 2005), these traits contribute to this group being a preferred species to culture, and contributes to their high consumer

acceptability. Despite all these positive attributes of tilapia, early maturity and easy spawning predispose the species to overcrowding and subsequent stunting of growth in mixed sex culture systems (Mair *et al.*, 1997; Piferrer, 2001). To overcome the negative impact of the precocious breeding strategy of tilapia, an alternative approach is to manipulate the gender of tilapia stock to obtain all-male populations. As the production of mono-sex tilapia becomes highly desirable in the aquaculture industry, efforts are being made to substitute the synthetic hormones employed in the sex reversal of genetic females to phenotypic males with less harmful plant phytochemicals. Pawpaw (*Carica papaya*) is one of the plants whose seeds have generated scientific research interests and are being considered as a potential agent for sex reversal, and the inhibition of reproductive activities in tilapia (Abdelhak *et al.,* 2013; Ampofo-Yeboah, 2013) in food fish production. Seeds of pawpaw contain some bioactive compounds such as benzyl isothiocyanate, benzyl thiourea, hentriacontane, β-sitosterol, caricin and oleanolic glycoside (Jegede & Fagbenro, 2008; Krishna *et al.*, 2008) which have the capacity to inhibit steroidogenesis thereby, leading to reproductive impairment (Lakshman & Changamma, 2013). Phytochemicals , also referred to as phytoestrogens, can modulate the hormonal systems to the extent that they are regarded as endocrine disrupting compounds (Rearick *et al.*, 2014). Endocrine disrupting compounds apart from their effect on the endocrine and reproductive systems, also affect other physiological functions of fish (Casanova-Nakayama *et al.*, 2011). Haematological parameters seems to be the most reliable indicator of alteration in the physiology of fishes (Akinrotimi *et al.*, 2012). This is because blood participates directly or indirectly in almost all biochemical processes in the body such as homeostasis as well as disease processes (Zorriehzahra *et al.*, 2010). In a nutshell, it is a reliable indicator of systemic response of fish to external stimuli (Tavares-Dias & Moraes, 2007).

Indices such as red and white blood cell counts, packed cell volume and haemoglobin concentrations are all subject to variations in stressful conditions such as disease (El-Feki *et al.*, 2000), environmental conditions like water temperature (Mali & Chavan, 2014), dissolved oxygen, pH (Das *et al.*, 2006), salinity (Salati *et al.*, 2010), high stocking density (Enache *et al.*, 2011), and contaminants (Affonso *et al.*, 2002). Also changes in blood parameters have the potential to be used as an indicator of variation in biochemical processes due to xenobiotic treatments. It is believed that changes of blood constituents could adversely affect the performance of the fish. Haematological parameters are also influenced by such factors as age (Charoo *et al.*, 2014), species (Fazio *et al.*, 2012), sex (Charoo et al., 2013) and sexual maturity (Santos *et al.*, 2009) of the fish. Conventionally, assay of blood cell counts (RBC and WBC), packed cell volume (PVC) and haemoglobin are undertaken using Neubauer haemocytometer, micro-haematocrit and cyanomethaemoglobin methods respectively, but because of advancement in science, automated systems have gained prominence especially in human and other mammalian blood assays. However, the use of automated systems in the assay of fish blood cells is not widespread because

unlike those of mammals, fish blood cells are nucleated (Hrubec *et al*., 2000). Even though the nucleated nature of fish blood makes calibration and assay using automated systems difficult, nonetheless a comparison of values obtained using automated systems and manual counting using haemocytometer shows no significant difference (Fazio *et al.*, 2012).

Apart from haematological parameters, analysis of blood chemistry offers information on biochemical changes in the blood and tissues which are vital for accurate diagnosis of infection or effect of treatment (Patriche *et al.*, 2011). Changes in blood chemical parameters are indicators of stress, health (Fazio *et al.*, 2012) and the nutritional status (Peres *et al.*, 2014) of fish. Total protein, albumin and Ca^{2+} are used to test for liver function and calcium metabolism (Zhou *et al*., 2009). There is no literature available presently on the effect of PSM on the haematological parameters and blood chemistry profile of *O. mossambicus*.

The purpose of this study was to evaluate the effect of the phytoestrogens contained in pawpaw seeds on the haematological and biochemical parameters of *O. mossambicus*. It also investigated the impact of dietary inclusion of PSM on the morphology and histology of the liver of the fish since the organ takes part in haematopoiesis and also detoxification of xenobiotic.

6.3 Materials and Methods

6.3.1 Experimental location and facilities

The experiment was conducted in a one tier plastic water re-circulatory system built in a glass house at the Aquaculture unit of the Welgevallen experimental farm of the University of Stellenbosch. The plastic tanks have a dimension of 70x40x38cm (LxWxH) and volume of 90 L. It also has aeration, filtration and water heating facilities incorporated into its design. The physicochemical parameters of the culture water were monitored daily. The mean water temperature recorded was 26.03 ± 0.61^0C, dissolved oxygen was 6.77 ± 0.78 mg/l while pH and conductivity were 6.42 ± 0.18 and 210 ± 8.02 respectively, please refer to Chapter 3 for more details.

6.3.2 Experimental animals and husbandry

A total of 1 000 juvenile *O. mossambicus* with an average weight of 24.81±8.54 g, and mean total and standard lengths of 11.06±1.30 cm and 8.84±1.14 cm, respectively, were obtained from Rivendell Hatchery, Grahamstown, South Africa. Upon arrival at the Welgevallen Experimental Farm of the University of Stellenbosch, the fish were acclimatized for three weeks in a holding facility before the commencement of the experiment. During the acclimatization period they were fed *ad-libitum* twice daily with commercial tilapia diet (Aquanutro,

Nutroscience (Pty) Ltd, Malmesbury, South Africa). The fish were fed *ad libitum* three times a day (9.00 – 9.30h, 13.00 – 13.30h and 17.00 – 17.30h) with the experimental diets. Uneaten food were syphoned out and the tanks cleaned every day with 50% water change. Daily monitoring of the water quality parameters were also undertaken.

6.3.3 Experimental diets

Fresh seeds were obtained from large quantities of ripe *C. papaya* obtained from fruit vendors in Stellenbosch, Western Cape, and dried in-doors. The dried seeds were blended to a fine powder using a laboratory grinder (Knifeter 1095, FOSS), and stored in Ziploc bags for later use. The standard (basal) diet consisted of a commercial tilapia diet (40% crude protein, Aqua-Nutro, Nutroscience (Pty) Ltd, Malmesbury, South Africa). The pawpaw seed meal (PSM) was added to the basal diets according to the inclusion level for that treatment group. A measured quantity of the basal diet and the experimental pawpaw seed powder were mixed thoroughly in Macadams baking system (model: SM-401). To enable pelleting of the feed, lukewarm water (200 mL/kg of feed) was added to the mixture during mixing. The mixture was pelleted in an extruder and oven-dried in a CFW Envirowatch 5 (model: Ø560) oven, and then stored in airtight containers till later use.

6.3.4 Experimental design

There were five experimental treatment groups with four replicates (5 X 4 factorial design). Each of the replicates had a stocking density of 50 fish. The treatment groups and the duration of the treatment were as shown in table 6.1.

Table 6.1 The inclusion level of pawpaw seed meal (PSM) in the diet of *O. mossambicus* and designation of treatment groups.

Treatment	Inclusion level of PSM	Designation
Basal diet (BD; negative control) for 2 months	No inclusion	BDM2
BD + PSM for 1 month	10 g PSM/ kg BD	P10M1
	30 g PSM/ kg BD	P30M1
BD + PSM for 2 months	10 g PSM/ kg BD	P10M2
	30 g PSM/ kg BD	P30M2

The treatment groups include the control that were fed only basal diet for 60 days (BDM2) and four groups fed 10 and 30 g PSM/ kg of BD for 30 and 60 days respectively. The groups fed the diet containing PSM for 30 days, were fed the BD from day-31 to end of the experiment.

6.3.5 Data recorded

On the day of stocking, the entire fish in the whole treatments and replicates were weighed individually and their total and standard lengths measured using weighing balance and meter rule respectively.

Length – weight measurement: The total and standard lengths of the specimen were measured using a measuring board graduated in centimeters. The total length was measured from the anterior most extremity of the fish to the end of the tail fin to the nearest centimeters. The standard length was measured from the most anterior extremity to the base of the tail fin. The base was identified by a crease when the tail was bent sharply from side to side. Top loading balance (Electronic Balance, UWE, HGS-300, capacity: 300 x 0.01g, Serial # P9440) was used to measure the body weight of the fish samples to the nearest grams. The weight of the dissected liver was also measured.

The hepatosomatic index (HSI) was calculated using the following formula; HSI = 100X (liver weight/body weight).

Blood analysis

Blood samples were collected from four fish (2 males and 2 females) from each replicate on Day 0, 30 and 60, respectively, for the determination of the haematological parameters and blood chemistry assay. The haematological parameters analysed included the red blood cell (RBC) counts, haemoglobin (HGB), packed cell volume (haematocrit, HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), thrombocytes (platelets, PLT), white blood cell (WBC) counts, neutrophils (NEU), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS), basophils (BASO). The blood chemical parameters analysed include cholesterol, total protein, albumin, globulin, albumin/globulin ratio, and glucose.

Two sets of blood samples were collected per fish during each sampling session. For the haematological assay, heparinized blood was collected and the assay undertaken as described in Chapter 3. The serum used for the chemistry assay were obtained from blood collected without anticoagulant, the method of collection and the assay were also described in Chapter 3.

6.3.6 Histological evaluation of tissue samples

At the end the experimental periods, histology of the liver was undertaken to determine the effects of the phytochemicals on their architectural integrity. Fish samples from each replicate (two males and two females) were randomly selected, weighed, humanely sacrificed and the abdomen incised and the liver dissected out.

After taking the weight the liver was fixed in 10% buffered formalin for the preparation of histological slides. The histological protocol was described in Chapter 3.

6.3.7 Statistical Analysis

The results are presented as mean±SE. The data were analysed by one way analysis of variance (ANOVA) with confidence interval of 95%. Variant means were separated using the Bonferroni (Dunn) t test. All statistical analysis was performed using XLSTAT version: 2015.2.02.18165.

6.4 Results

The body characteristics of the *O. mossambicus* fed different levels of PSM and the control are presented in Table 6.2. The mean weight ranged from the maximum (48.36±1.58) in P10M2 to the minimum (44.24±1.82) in P30M2, but the groups did not differ. The mean lengths (total and standard) of the treatment groups also did not differ significantly.

Table 6.2 Morphometric characteristics (mean±SE; Range) of *O. mossambicus* fed diets containing 10 and 30 g PSM during 60 culture period –please rephrase title of table. See changes I suggested in previous version.

Treatment	Total length (cm)	Standard length (cm)	Weight (g)
BDM2 (Control)	13.75±0.17 (9.1–19.3)	10.8±0.14 (6.7–15.7)	44.56±1.72 (9.45–114.5)
P10M1	13.82±0.18 (9.2–19.5)	10.86±0.13 (7.2 – 15.5)	46.25±1.74 (12.15–124.03)
P30M1	13.65±0.17 (9.3–18.7)	11.1±0.16 (7.5 – 15.7)	45.7±1.72 (13.2–104.75)
P10M2	13.93±0.19 (9.3–19.9)	11.21 ± 0.17 (7.2 – 17.0)	48.36±1.58 (12.65–115.9)
P30M2	13.64±0.21 (9.8–19.8)	10.71 ± 0.13 (7.8 – 15.5)	44.24±1.82 (14.75–120.75)

6.4.1 Haematological parameters

The respective haematological parameters of *O. mossambicus* fed various levels of pawpaw seed meal at the days 0 and 30 are shown in Tables 6.3 and 6.4 respectively, while that of day 60 are presented Table 6.5. No differences were observed for the fourteen respective haematological indices assayed for samples collected on Day 0 of the study (Table 6.3). At day 60, the maximum mean RBC (2.26±0.05x10^{12}/L) recorded in P10M1 group while the minimum (2.15±0.05x10^{12}/L) recorded in P30M1 group. The maximum haemoglobin (12.79±0.3) was recorded in BDM2 (control) group and the minimum (12.07±0.3) in P30M1.

117

Table 6.3 Haematological parameters (mean±SE) of *O. mossambicus* fed 10 and 30 g of PSM/ kg of BD at Day 0.

Parameter	P30M2	P30M1	P10M2	P10M1	BDM2
Red blood cell (10^{12}/L)	1.58±0.2	1.50 ± 0.1	1.48±0.1	1.53±0.2	1.47±0.1
Haemoglobin (g/dL)	9.04±0.4	8.52±0.5	8.11±0.5	8.94±0.6	9.17±0.5
Haematocrit (%)	23.10 ± 1.8	21.67±1.7	21.39±1.7	22.57±1.9	22.64±1.7
Mean corpuscular volume (fL)	152.13±2.6	155.5±3.1	153.69±2.7	149±2.6	155.81±2.9
Mean corp. haemoglobin (pg)	53.83±0.8	54.16±0.7	52.71±0.9	53.94±0.8	54.04±0.7
Mean corp. haem. concn (g/dL)	35.68±0.6	34.88±0.6	34.44±0.8	36.18±0.5	34.64±0.6
Red cell distribution width (%)	12.16±0.5	12.34±0.8	11.19±0.6	11.66±0.5	11.62±0.7
Platelets (10^9/L)	26.85±5.8	47.11±4.7	46.45±5.7	24.0±4.9	46.66±5.6
White blood cell (10^9/L)	1.11±0.4	1.14±0.5	0.86±0.4	2.1±0.7	1.32±0.4
Neutrophils (% of WBC)	6.56±2.2	7.3±2.3	10.55±2.6	11.65±2.4	9.74±2.3
Lymphocytes (% of WBC)	76.27±3.3	75.1±3.5	73.69±3.5	76.93±3.7	70.63±3.4
Monocytes (% WBC)	9.54±2.4	11.27±2.3	10.11±2.6	6.29±2.4	12.25±2.5
Eosinophils (% of WBC)	5.44±1.0	4.05±0.9	3.78±1.1	4.04±1.0	4.75±0.8
Basophils (% of WBC)	2.2±0.8	2.27±0.7	1.87±0.7	1.09±0.6	2.33±0.5

The maximum mean haematocrit (34.27±0.78) was recorded in P10M1 while the minimum (32.27±0.78) was recorded in P30M1. The maximum WBC count (2.45±0.29x10^9/L) was recorded in P30M1 and the minimum (1.72±0.29x10^9/L) was obtained in P10M2. There was also no significant difference in the haematological parameters recorded on the days 30 and 60. There was no distinct trend of increase or decrease in the values of the haematological parameters that can be ascribed to the effect of the pawpaw seed meal.

Table 6.4 Haematological parameters (mean±SE) recorded for blood samples collected on Day 30 from *O. mossambicus* that received diets that contained respectively 10g and 30 g PSM/kg BD.

Parameter	P30M2	P30M1	P10M2	P10M1	BDM2
Red blood cell (10^{12}/L)	2.22±0.2	1.91±0.1	2.29±0.1	2.17±0.2	2.24±0.1
Haemoglobin (g/dL)	12.39±0.6	10.66±0.5	12.64±0.4	12.11±0.6	12.72±0.5
Haematocrit (%)	33.18±1.1	29.08±1.1	34.88±1.2	34.05±1.2	34.15±1.3
Mean corpuscular volume (fL)	150.2±1.7	153.3±1.8	152.6±1.6	149.6±1.7	152.6±1.6
Mean corp. haemoglobin (pg)	56.12±0.6	55.89±0.5	55.23±0.5	55.66±0.6	56.79±0.7
Mean corpuscular haem. Concn. (g/dL)	37.45±0.6	36.49±0.4	36.25±0.5	37.24±0.6	37.24±0.5
Red cell distribution width (%)	13.36±0.7	13.75±0.6	12.61±0.6	13.77±0.5	13.55±0.6
Platelets (10^9/L)	26.81±5.4	28.77±6.4	20.15±6.8	26.23±6.3	37.59±6.1
White blood cell (10^9/L)	1.88±0.2	1.66±0.1	2.11±0.1	1.74±0.2	2.39±0.2
Neutrophils (% of WBC)	4.31±1.1	4.23±1.0	3.28±1.0	5.21±0.9	2.44±1.0
Lymphocytes (% of WBC)	86.73±2	86.95±1	85.08±2	80.89±3	88.65±2
Monocytes (% WBC)	6.21±1.6	5.12±1.5	8.29±1.6	9.53±1.7	6.12±1.5
Eosinophils (% of WBC)	1.85±0.5	2.45±0.6	1.85±0.6	2.15±0.5	1.08±0.7
Basophils (% of WBC)	0.91±0.7	1.25±0.6	1.46±0.5	2.22±0.7	1.71±0.6

Table 6.5 Haematological parameters (mean±SE) of *O. mossambicus* fed 10 and 30 g of PSM/ kg of BD at Day 60.

	P30M2	P30M1	P10M2	P10M1	BDM2
Red blood cell (10^{12}/L)	2.19±0.2	2.15±0.1	2.22±0.1	2.26±0.1	2.23±0.2
Haemoglobin (g/dL)	12.12±0.5	12.07±0.3	12.51±0.3	12.77±0.4	12.79±0.3
Haematocrit (%)	32.27±0.8	32.36±0.7	33.44±0.8	34.27±0.8	34.22±0.9
Mean corpuscular volume (fL)	147.4±1.6	150.1±1.8	151.1±1.6	152.0±2.1	154.1±1.6
Mean corp. haemoglobin (pg)	55.34±0.6	55.88±0.7	56.27±0.6	56.56±0.5	57.47±0.6
Mean corpuscular haem. Concn. (g/dL)	37.63±0.5	37.26±0.5	37.34±0.4	37.28±0.5	37.4±0.6
Red cell distribution width (%)	12.43±0.4	12.49±0.6	12.82±0.5	12.53±0.6	12.18±0.5
Platelets (10^9/L)	6.43±1.4	8.66±1.3	5.35±1.3	6.23±1.6	6.82±1.5
White blood cell (10^9/L)	2.13±0.3	2.45±0.2	1.72±0.3	1.79±0.4	1.91±0.3
Neutrophils (% of WBC)	1.72±0.6	3.74±0.6	3.16±0.5	2.42±0.6	2.97±0.5
Lymphocytes (% of WBC)	84.68±1.6	82.42±1.7	79.89±1.6	82.22±1.5	82.39±1.6
Monocytes (% WBC)	9.16±2.3	13.37±2.3	11.55±2.6	10.56±2.3	10.71±2.5
Eosinophils (% of WBC)	2.14±0.6	2.86±0.6	2.38±0.7	2.40 ± 0.5	2.57±0.6
Basophils (% of WBC)	2.3±0.5	2.02±0.4	3.03±0.4	2.33±0.5	1.36±0.4

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Influence of gender on the haematological parameters

The mean red blood cell (RBC) count, haemoglobin, haematocrit and mean corpuscular haemoglobin concentration (MCHC) of *O. mossambicus* was significantly higher in males than in females, while white blood cell (WBC) count, mean corpuscular volume (MCV) was significantly higher in females than in males. The mean corpuscular haemoglobin (MCH), red cell distribution width (RDW) and differential counts (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) were not significantly different between the sexes as shown in Table 6.6.

Table 6.6 Influence of gender on the haematological parameters (mean±SE) of *O. mossambicus.*

Parameters	Males	Females
Red blood cell (10^{12}/L)	2.39±0.0[a]	2.02±0.1[b]
Haemoglobin (g/dL)	13.5±0.2[a]	11.41±0.2[b]
Haematocrit (%)	35.3±0.4[a]	31.32±0.5[b]
Mean corpuscular volume (fL)	147.55±1.0[b]	154.3±1.0[a]
Mean corp. haemoglobin (pg)	56.39±0.4[a]	56.1±0.5[a]
Mean corpuscular haem. Concn. (g/dL)	38.29±0.2[a]	36.47±0.3[b]
Red cell distribution width (%)	12.52±0.3[a]	12.46±0.3[a]
Platelets (10^9/L)	6.04±0.8[a]	7.36±0.7[a]
White blood cell (10^9/L)	1.47±0.2[b]	2.53±0.2[a]
Neutrophils (% of WBC)	2.9±0.4[b]	2.71±0.3[a]
Lymphocytes (% of WBC)	83.28±1.0[a]	81.36±1.0[a]
Monocytes (% WBC)	11.32±1.5[a]	10.82±1.2[a]
Eosinophils (% of WBC)	2.04±0.3[a]	2.91±0.4[a]
Basophils (% of WBC)	2.2±0.3[a]	2.21±0.5[a]

[a,b] *Rows with different superscripts differ significantly (P < 0.05)*

6.4.2 Blood serum chemistry

The blood serum chemistry profile of *O. mossambicus* juveniles fed 10 and 30 g of PSM/kg of BD and the control at days 30 and 60 post exposure [Table 6.7].

Cholesterol (mmol/L): The maximum (mean+SE) level of cholesterol in the serum of experimental fish at day 60 post exposure (9.86±0.56) was recorded in BDM2 group and the minimum (6.05±0.54) recorded in P30M1. The

120

differences in the cholesterol levels between different treatment groups were not significant at day 30 and 60 post exposure.

Total protein (g/L): The maximum and minimum values of total protein were 50.6±1.4g/L (BDM2) and 47.8±1.5g/L (P10M2 and P10M1) respectively at day 60. The values were not significantly different between treatment groups.

Albumin (g/L): There was also no significant difference in the albumin fraction of the serum protein between different treatment groups. The maximum mean albumin concentration (15.2±0.7g/L) was recorded for P30M2 and the minimum (13.9±0.7) recorded for P30M1.

Table 6.7 Blood chemistry (Mean$\pm$SE) of *O. mossambicus* fed 10 and 30 g of PSM for 30 and 60 days.

Parameter	Feeding period of PSM	BDM2	P10M1	P30M1	P10M2	P30M2
Cholesterol	Day 30	9.86$\pm$0.56	6.64$\pm$0.54	6.05$\pm$0.54	7.29$\pm$0.53	6.85$\pm$0.54
(mm/L)	Day 60	17.39$\pm$1.45	13.99$\pm$1.57	14.16$\pm$1.45	14.20$\pm$1.50	13.36$\pm$1.57
Total protein (g/L)	Day 30	47.79$\pm$1.80	44.29$\pm$1.74	42.96$\pm$1.74	41.06$\pm$1.68	39.81$\pm$1.74
	Day 60	50.60$\pm$1.40	47.80$\pm$1.50	48.60$\pm$1.40	47.80$\pm$1.50	49.4$\pm$1.40
Albumin (g/L)	Day 30	14.57$\pm$0.90	12.69$\pm$0.96	11.43$\pm$0.92	11.44$\pm$0.89	11.43$\pm$0.95
	Day 60	14.20$\pm$0.68	14.90$\pm$0.70	13.90$\pm$0.70	14.40$\pm$0.72	15.2$\pm$0.70
Globulin (g/L)	Day 30	33.07$\pm$1.07	32.31$\pm$1.08	31.54$\pm$1.04	29.67$\pm$1.00	28.36$\pm$1.07
	Day 60	36.40$\pm$1.02	33.00$\pm$1.01	34.60$\pm$1.00	33.34$\pm$1.00	34.2$\pm$1.00
Albumin/ Globulin	Day 30	0.44$\pm$0.03	0.37$\pm$0.04	0.35$\pm$0.03	0.39$\pm$0.02	0.39$\pm$0.03
ratio	Day 60	0.39$\pm$0.02 [b]	0.45$\pm$0.02 [ab]	0.40$\pm$0.03 [ab]	0.43$\pm$0.03 [ab]	0.46$\pm$0.02 [a]
Glucose (mmol/L)	Day 60	27.90$\pm$3.10	34.5$\pm$3.30	24.60$\pm$3.10	27.60$\pm$3.80	25.4$\pm$3.10

[a,b] *Rows with different superscripts differ significantly (p<0.05)*

Globulin (g/L): The maximum value of 36.4±1.0g/L was recorded for BDM2 (control) while the minimum 33.0±1.0g/L was obtained in P10M1. The differences between treatment groups were statistically non-significant.

Albumin/Globulin ratio: The derived albumin to globulin ratio obtained in this study shows the highest mean value of 0.46±0.02 recorded for P30M2 group while the lowest mean value of 0.39±0.02 was obtained among

the control (BDM2) group. This difference in the mean value of the group fed 30 g of PSM /kg of BD for 60 days (P30M2) was significantly higher than that of the control group fed only basal diet (BDM2) as shown in Table 6.7.

Glucose (mmol/L): The maximum mean value of glucose concentration (34.5±3.3mmol/L) was obtained in P10M1 group while the minimum (24.6±3.1mmol/L) was recorded for P30M1. The differences between groups were not significant (p>0.05).

Influence of gender on the blood chemistry profile

The blood chemistry assay indicated that the mean levels of cholesterol, total protein, albumin, and also the derived albumin to globulin ratio were significantly higher in female *O. mossambicus* than in males (Table 6.8). Also the mean globulin level of females (36.23±0.63) was significantly higher than those of males (32.38±0.60). Conversely, the glucose level in males (37.44±1.94) was significantly higher than those of the females (18.59±2.04) as shown in Figure 6.3.

Table 6.8 Influence of gender on the blood chemical parameters (mean$\pm$SE) of *O. mossambicus.*

Parameter	Males	Females
Cholesterol	10.83±0.92[b]	18.42±0.96 [a]
Total Protein	44.08±0.88 [b]	53.56±0.92 [a]
Albumin	11.70±0.41 [b]	17.34±0.41 [a]
Globulin	32.38±0.60 [b]	36.23±0.63 [a]
Albumin/Globulin ratio	0.37±0.01 [b]	0.48±0.01 [a]
Glucose	37.44±1.94[a]	18.59±2.04 [b]

[a,b] *Rows with different superscripts differ significantly (p<0.05)*

6.4.3 Liver weight and hepatosomatic index

Results of the mean liver weight from *O. mossambicus* exposed to various levels of pawpaw seed meal shows the maximum value (1.48±0.13 g) in BDM2 group followed by P30M2 (1.21±0.13 g), while the minimum (0.85±0.13 g) was in P10M1 (Table 6.6). The fish that received 10 g of PSM per kg BD for 30 days had a significantly lighter mean liver weight as shown in Table 6.9.

Result of the hepatosomatic index (HSI) of the experimental fish from the different treatment groups shows the maximum mean value (2.21±0.16) recorded among the group that received 10 g/kg of the experimental diet for 30 days. However, the differences in the HSI among the groups were not statistically significant (p>0.05).

Table 6.9 Effect of C. papaya on the liver weight and hepatosomatic index (Mean±SE)

Treatment	Body weight (g)		Liver weight (g)	Hepatosomatic index
	Male	Female		
P30M2	103.58±3.38	46.08±3.42	1.21±0.13 [a]	1.61±0.18
P30M1	102.51±3.44	45.41±3.58	1.17±0.12 [a]	1.46±0.17
P10M2	95.25±3.48	45.71±3.44	1.05±0.14 [a]	1.53±0.16
P10M1	92.5±3.46	46.78±3.48	0.85±0.13 [b]	1.2±0.17
BDM2	94.82±3.48	48.58±3.45	1.48±0.14 [a]	2.21±0.16

[a,b] *Rows with different superscripts differ significantly (P<0.05)*

Influence of gender on liver weight and hepatosomatic index

The mean liver weight of male *O. mossambicus* was significantly higher than that of the females, whereas the hepatosomatic index of the females were higher than those of the males though the difference was not statistically significant [Figure 6.1].
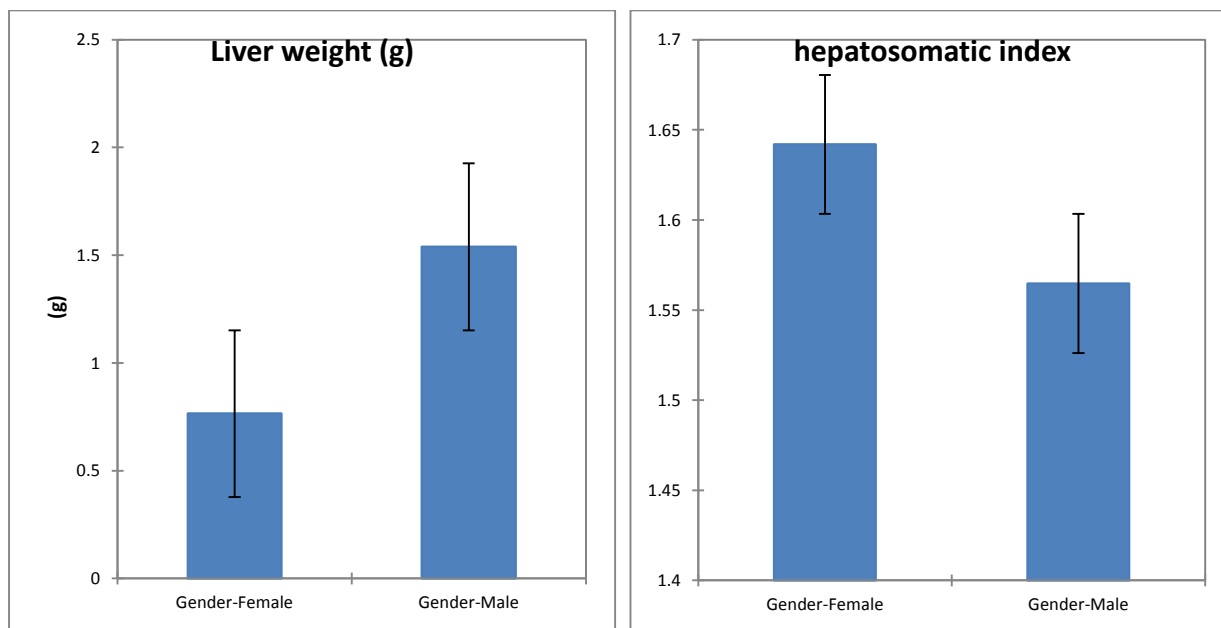
Figure 6.1 Influence of gender on liver weight and hepatosomatic index of *O. mossambicus*

6.4.4 Histopathological findings

Qualitative analysis of the histological slides showed minor alterations in the liver of the group fed 30 g of PSM per kg of BD for 60 days while no pathology was observed in the control and low dose group. The only histopathological lesion observed were degeneration and vacuolization of the hepatocytes in less than 10% of the members of the group fed 30 g of PSM/ kg of basal diet for 60 days contrary to control and the group fed 10 g PSM per kg BD which had normal liver as shown in Figure 6.2.
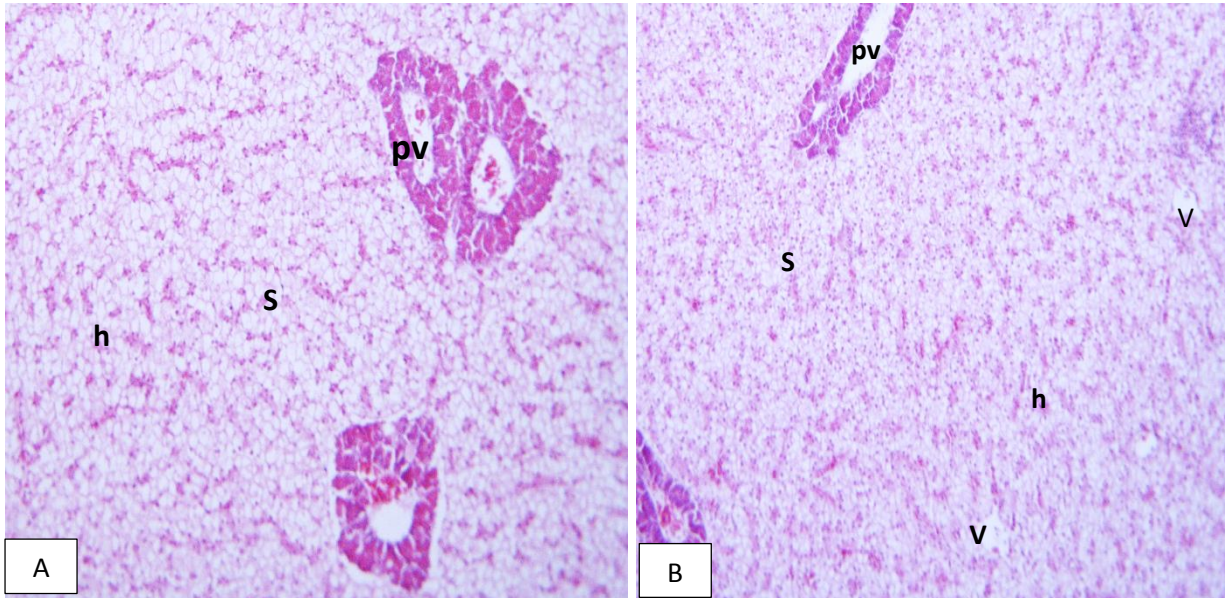
Figure 6.2 Liver of *O. mossambicus* from control (A) and group fed 30 g of PSM/kg of basal diet for 60 days indicating hepatocytes (h), portal vein (pv), sinusoid (S), vacuolization of the hepatocytes (V) (H&E staining, 100X magnification)

6.5 Discussion

Blood parameters such as RBC, WBC, PCV, haemoglobin and erythrocyte indices (MCV, MCH and MCHC) are indicators of toxicity such as those due to xenobiotic treatments and organophosphate insecticides, chlorpyrifos (Muttappa *et al.*, 2015). Evaluation of the haematological profile of fish blood is important in aquaculture for the determination of the health status, impact of treatment and experimental procedures. In this study, fish received 30 and 10 g PSM/kg of BD for 30 and 60 days, and the RBC, WBC, PCV, haemoglobin and erythrocyte indices (MCV, MCH and MCHC) and differential WBC (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) were analysed to determine the effect of PSM on liver function and blood parameters.

124

The result of RBC counts in this study between different treatment groups at day 60 ranged between maximum (2.26 ± 0.05 X10^{12}/L) obtained in the group that were fed 10 g/kg PSM for 30 days (P10M1), while the minimum (2.15 ± 0.05 X10^{12}/L) was from the group that were fed 30 g/kg of the PSM for 30 days. The control group had mean RBC count of 2.23 ± 0.05 X10^{12}/L. There was reduction in the level of RBC among the treatment groups that were fed 30 g/kg diet as shown in Figure 6.1, however the difference is not statistically significant which means that PSM may not affect the erythrocyte count at an inclusion level of 30 g/kg. However, this is contrary to the findings of Ayotunde *et al.* (2010) who reported significant decrease in RBC counts of *Clarias gariepinus* fingerlings exposed to various levels of pawpaw seed extract ranging from 100 to 500 mg/20L of the culture water. Also Kavitha *et al.* (2012) reported a decreased RBC in Common carp (*Cyprinus carpio*) treated with 12.40mg/L of *Moringa oleifera* seed extract*.* Lohiya *et al.* (2005), observed no influence of the pawpaw seed on RBC count of albino rats treated with 50 mg of pawpaw seed extract per kg body weight per day. Red blood cells (erythrocytes) contain haemoglobin and its main function is the transport of oxygen and carbon dioxide and abnormal changes in its number may indicate anaemia or stress (De Pedro *et al.*, 2005). It is the most abundant cell type and has proved to be a highly variable blood parameter among fish species (Daneshvar *et al.*, 2012). Elahee & Bhagwant (2007) found the RBC to range from 0.97X10^6/ mm^2 in *Siganus sutor* to 3.39 X10^6/ mm^2 in *Epinephalus merra* when comparing counts from three species. Other reported values include 3.53 X10^6/μL (range: 2.21 – 4.47 X10^6/μL) for grey mullet (*Mugil cephalus*) (Fazio *et al.*, 2012). Hrubec *et al*. (2000) gave a reference interval for RBC counts in tilapia (*Oreochromis* hybrid) of 1.91 – 2.83 X10^6/μL.

Gender is one of the factors believed to influence the haematological parameters of fish species (Santos *et al.*, 2009). The mean RBC count of male *O. mossambicus* (2.39 X10^{12}/L) was significantly higher than that of the females (2.02 X10^{12}/L) in this study. This observation agrees with the finding of Karimi *et al.* (2013) who reported significant higher RBC count in male yellow fin sea bream (*Acanthopagrus latus*) compared to the females. It has been postulated that significantly higher RBC, haematocrit and haemoglobin in male fish may be due to higher metabolic rate (Acharya & Mohanty, 2014), since RBC determines the dissolved oxygen carrying capacity of the blood. This is however, contrary to the report of (Santos *et al.*, 2009) who stated, that there was no difference in the haematology of male fat snook (*Centropomus parallelus*) with those of the females.

Packed cell volume (PCV; haematocrit) values obtained in this study between treatment groups had the maximum (34.27 ± 0.78%) obtained in the group fed 10 g/kg of PSM for 30 day (P10M1), while the minimum (32.27 ± 0.78%) in the group fed 30 g/kg for 60 day (P30M2). The control group (BDM2) recorded mean PCV of 34. 22 ± 0.78%, however the differences between treatment groups were not significant even though the groups that were fed higher PSM (P30M2 and P30M1) had lower PCV than the control as shown in Figure 6.1. In

laboratory animal research (Lohiya *et al.*, 2005b), reported no significant influence of the pawpaw seed extract on the haematocrit values of albino rat. Clark *et al.* (1979) stated that haematocrit values for fish usually vary between 20 – 35% from the mean value reported, with variation rarely exceeding 50%. The mean haematocrit values reported in this study falls within the range 20 – 35% reported by Clark *et al.* (1979). In this study, the males had significant higher (35.3%) haematocrit values than females (31.32%). Differences in haematocrit values between the sexes have been reported for sexually mature yellow seabream (*Acanthoparus latus*) (Karimi *et al.*, 2013) who reported mean haematocrit for males (31.18%) and females (29.07%).

This study recorded a range of haemoglobin values of 12.07 g/dL for P30M1 (fed 30 g PSM/ kg diet for 30 days) and 12.79 g/dL for BDM2 (control). These data fall within the range of values of haemoglobin reported by (Chuku & Uwakwe, 2012) who reported a range of 3.7 to 12.94 g/dL (mean: 10.28±2.7 g/dL) for tilapia species. Even though the group that were fed higher inclusion levels of PSM had lower mean haemoglobin than the control, the difference was not statistically significant therefore, it can be inferred that PSM has no effect on the haemoglobin up to an inclusion level of 30 g/kg diet. However, this is contrary to (Ayotunde & Ofem, 2008) who reported significant decrease in haemoglobin of *O. niloticus* exposed to a concentration of 5.0 mg of pawpaw seed extract per litre of culture water. The species, dose and mode of administration of the PSM may have been the cause of the difference in haemoglobin obtained in this study with that of Ayotunde & Ofem (2008). Haemoglobin values are one of the haematological parameters mostly used for evaluating fish health. Haemoglobin profile is indicative of oxygen carrying capacity of the blood and anaemia is indicated by low haemoglobin (Zhang *et al.*, 2007). At an inclusion level used in this study, PSM is not potent enough to induce anaemia in *O. mossambicus* and therefore it can be said to be safe for use in tilapia culture.

The erythrocyte indexes; mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) recorded in this study were within the range reported by Lucas *et al.* (2003) who recorded range of MCV (12.36 – 528.57 /μ), MCH (5.07 - 120.86 pg) and MCHC (19.84 – 87.73%) for Nile tilapia (*Oreochromis niloticus*). Even though at higher inclusion level of PSM (30 g/kg) in treatments P30M2 and P30M1 groups recorded reduced MCV and MCH compared with the control (Figure 6.1), the differences were not significant. The changes induced on the MCHC by the PSM were also not statistically significant.

White blood cell (leucocytes) count in fish blood varies according to season, age and sex (Hrubec *et al.*, 2001; Charoo *et al.*, 2014). Leucopoenia or leucocytosis are pathological conditions associated with abnormal leucocyte count indicating the possibility of alteration in immune function of the individual (De Pedro *et al.*, 2005). A

response of the cellular immune system to infection or treatments manifests as an increase in WBC count (Rapatsa & Moyo, 2013). Fazio *et al.* (2013) presented data on the mean WBC count for several species of fish, ranging from 0.94±0.14 X10^9/L for *Gobius niger* to 4.74±0.9 X10^9/L for *Sparus aurata*. The WBC count in this study between treatment groups ranged from maximum (2.45 X10^9/L) obtained in P30M1 (fed 30 g PSM/ kg diet for 30 days) to minimum (1.72 X10^9/L) obtained in P10M2 (10 g PSM/ kg diet for 60 days). There was no significant difference in the mean WBC count of the different treatment groups indicating no influence of PSM on the respective parameters up to an inclusion level of 30 g/ kg. This result agrees with the report of Ozovehe (2012) who reported no influence of *Moringa oleifera* on the haematology of *Clarias gariepinus*. Apart from absolute count, differential WBC counts are equally very important in disease diagnosis and determination of impact of treatment or experimental procedures on fish. White blood cells are grouped into granulocytes also called polymorphonuclear leucocytes (made up of neutrophils, eosinophils and basophils) and mononuclear leucocytes (granulocytes) that consist of lymphocytes and monocytes. Lymphocytes are the most abundant of the leucocytes in fish blood constituting more than 85% of the WBC while neutrophils is the most common type of granulocytes (Vázquez & Guerrero, 2007). Neutrophils (also referred to as heterophils) are phagocytic and play a key role in acute inflammation as a response to disease stimulus while lymphocytes play a key role in immune response (Claver & Quaglia, 2009). Although, Ayotunde *et al.* (2010) reported that PSM significantly decreased the level of WBC of *O. niloticus*, results from this study did not indicate a similar effect on the WBC count in *O. mossambicus*. In this study, the PSM also had no influence on the differential leucocyte counts (neutrophils, lymphocytes, monocytes, eosinophils and basophils). Lymphocytes were the most abundant of the leucocytes with a maximum percentage composition of 88.7% of the WBC recorded in BDM2 and minimum (80.9%) recorded in P10M1. On the other hand, basophils were the least in terms of percentage composition with its maximum (2.22%) obtained in P10M1 and the minimum (0.91%) in P30M2. These findings were in agreement with Antache *et al.* (2014) who reported that lymphocytes constitute 96.4% of the WBC of *O. niloticus* while neutrophils and monocytes were 1.73 and 1.06% respectively.

Thrombocytes (platelets) is second to erythrocytes in terms of abundance in fish blood where it plays key role in blood clotting (Vázquez & Guerrero, 2007). In this study, there was no significant difference in the mean number of thrombocytes between treatment groups, and also no distinct trends established in the values that can be attributed to the effect of pawpaw seed meal. Abnormal decreases in RBC, HGB, PCV and erythrocyte indices (MCV, MCH and MCHC) indicate anaemia (De Pedro *et al*. 2005) while, decreased WBC means reduced disease fighting capacity of the fish (Rapatsa & Moyo 2013). It has been suggested that, xenobiotic treatments that result in the increase of these blood parameters are associated with an improved immune system functioning, compared to treatments that result in their decrease, which are associated with a compromised immune

response (Anene *et al.*, 2015). The haematological parameters recorded in this study did not show that the immune system was depressed due to inclusion of pawpaw seed meal in the diet of *O. mossambicus.*

Serum biochemical parameters are indicators of the health status of animals including fish and it varies among fishes as a result of species, age, diet and sampling methodology (Celik, 2004; Akbary, 2014). Elevated levels of total protein may indicate structural alteration of the liver with concomitant reduction in deamination whereas hypo-proteinemia (decrease in total protein level) may be as a result of inhibition of protein synthesis in the liver (Al-Asgah, 2015). Total serum protein values in this study ranged from 50.6±1.4 g/L obtained in BDM2 (control) to 47.8±1.5 g/L in P10M2 (fed 10 g/kg diet for 60 days) and P10M1 (fed 10 g/kg for 30 days) respectively, with no significant differences reported between the treatment groups. The values reported in this study were within the range (2.8 – 6.0 g/dL) of total protein reported for shortnose sturgeon (*Acipenser brevirotrum*) by Knowles *et al.* (2006).

Blood protein has been used by scientists to determine the physiological conditions of test animals and has been thought to be one of the indicators of fish immune system (Demir *et al.*, 2014). According to Charoo *et al*. (2013) there is a tendency of the protein components of blood to increase when fish is stressed due to starvation. The total serum protein consist of mainly the albumin and globulin fractions (Upadhyay *et al*., 2014). The main function of the albumin fraction of serum protein is the transport of lipids (exogenous fatty acids and endogenous metabolites), and it is reported to increase when fish is exposed to toxicants (Upadhyay *et al*., 2014). A mean albumin concentration of 1.6±0.46 g/dL has been reported in *O. mossambicus* (Demir *et al.*, 2014). In this study the albumin concentration ranged from maximum (15.2±0.7 g/L) obtained in P30M2 while the minimum (13.9±0.7 g/L) was in P30M1. The difference between treatment groups was not significant which shows that the diet did not predispose the treated fish to osmoregulatory dysfunction. The globulin fraction of the serum protein are associated with innate response against external stimuli (disease, stress and treatment) in fish (Cnaani *et al.*, 2004). Globulins are made up of alpha, beta and gamma (α, β, and γ) globulins. Gamma globulins consist mainly of immunoglobulins and are synthesized in plasma cells.

The derived albumin to globulin ratio (A/G) of the group fed 30 g of PSM /kg of BD for 60 days (P30M2) was significantly higher than that of the control group fed the basal diet (BDM2) in this study. Kaleeswaran *et al*. (2012) reported an increased A/G ratio in Indian major carp (*Catla catla*) fed an ethanolic extract of *Cynodon dactylon*, while Sharafeldin *et al*. (2014) also reported an elevated A/G ratio in *O. niloticus* exposed to acute and chronic profenofos, an organophosphorus insecticide. However Hasheesh *et al*. (2011) reported no significance difference in the A/G ratio of *O. niloticus* treated with 17α-methyl testosterone while Kumar *et al*. (2011)

reported decreased A/G ratio in *O. mossambicus* exposed to endosulfan. Abnormal increases in A/G ratio may occur as a result of disturbances in liver function or failure to excrete albumin if the animal is stressed due to xenobiotic treatment (Sharafeldin *et al*., 2014). Elevated A/G ratio may be due to decrease in globulin fraction of the total protein. The implication of decreased level of globulin may be an indication of immune suppression since globulins are precursors of immunoglobulins which play a vital role in immune function of an organism.

Cholesterol concentrations in the study had no distinctive trend however the maximum (17.39±1.45 mmol/L) was in the BDM2 treatment while the minimum (13.36±1.57 mmol/L) was in P30M2, the differences between treatment groups were insignificant. Cholesterol concentrations are controlled by the liver through regulation of the lipoprotein metabolism therefore abnormal increase in the level of cholesterol may be due to disorders of lipoprotein metabolism. Cholesterol is the precursor of steroid hormones therefore sexual development and nutritional statuses are among the factors that determine its level in the blood (Patriche *et al.*, 2011). Cholesterol level was not affected by the experimental diet in this study. Glucose is the source of energy for the cellular metabolism and its levels are used as indicators of stress (Cnaani *et al.*, 2004) and nutritional status (Demir *et al.*, 2014) in fish. Hyperglycaemia which indicates increased level of blood glucose, is associated with impaired carbohydrate metabolism (Al-Asgah, 2015). This study did not establish any distinct trend in the level of glucose between treatment groups, indicating no adverse influence of PSM on the carbohydrate metabolism of the fish.

The biochemical profiles are influenced by the gender of the fish among other factors. The serum levels of cholesterol, total protein, albumin, and albumin/globulin ratio were significantly higher in female *O. mossambicus* than in males in all the treatment groups while the globulin level of females was significantly higher in all the treatment groups except P30M2 and P30M1. This finding was in agreement with findings of Acharya & Mohanty (2014), who reported higher values of the total protein, albumin, globulin, glucose and cholesterol in female *Clarias batrachus* and *Heteropneustes fossilis* than their male counterparts. However, this findings was contradicted by Charoo *et al*. (2013) who reported higher total protein, albumin and glucose in male rainbow trout (*Oncorhynchus mykiss*) than in the females in their study. Conversely, the glucose level in males was significantly higher than those of the females in all the treatment groups in the present study which agreed with the findings of Charoo *et al*. (2013).

The ratio of liver weight to body weight termed the hepatosomatic index (HSI) is valuable in the study of effect of treatments on fish species (Ighwela *et al*., 2012). Evaluation of the HSI makes it possible to identify pathological conditions such as atrophy, hypertrophy or hyperplasia associated with certain disease conditions and exposure to toxicants (Rearick *et al.*, 2014). The liver is the organ whose functions include the metabolism of

carbohydrates, proteins and fats, the storage of glycogen, vitamins and iron, and most importantly the detoxification of drugs and toxins. It is this latter function that makes the liver the target for attack by toxicants (Hachfi *et al.*, 2012). Results from the present study recorded liver weights of P10M1 (0.85±0.13 g) being significantly lower than those of the control and other treatment groups [Table 6.6]. However, there was no significant difference in the HSI between treatment groups in this study, in contrast, Ighwela *et al.* (2014) recorded an increased HSI in *O. niloticus* fed high levels of dietary maltose. The only tilapia data available on the effect of treatments is for Nile tilapia therefore this study present the first information on the effect of PSM on liver weight and HSI of *O. mossambicus*. To compare with the present study, however Rearick *et al.* (2014) reported no significant effect of three naturally occurring phytoestrogens (genistein, diadzein and formononetin) on the HSI of fathead minnows.

Accumulation and biotransformation of xenobiotics takes place in the liver, and certain xenobiotic chemicals can cause liver damage (Kandiel *et al.*, 2014). Liver being the primary organ of detoxification are prone to deleterious impacts of toxicants (Köksal *et al.*, 2008), therefore histological changes in the architecture of the liver can be used to assess the effect of xenobiotic/ chemicals in experimental fish. In this study, there were minor histopathological changes in the liver of less than 10% of the group fed 30 g PSM/ kg of basal diet for 60 days. The lesions observed included degeneration and vacuolization of hepatocytes, however these lesions were not observed among the group fed 30 g of PSM /kg of basal diet for 30 day signifying a possible reversible effect on withdrawal of treatment. It might also be that the liver had a short-term ability to handle level of phytoestrogens, but when the treatment period was extended, the levels in body increased to eventually compromise the liver's ability to process phytoestrogens. This is in agreement with the findings of Hossam & Wafaa (2011) who reported cellular vacuolization and swelling of hepatocytes in *O. niloticus* fed dietary PSM. Presently, there are no reports available on the possibility of PSM accumulating in the liver of *O. mossambicus*.

6.6 CONCLUSIONS

Results obtained in this study suggests that the dietary inclusion of PSM has no negative effects on the immune system of *O. mossambicus*, and that there is a possibility of including the seed meal up to 30 g/kg of basal diet of *O. mossambicus* to inhibit their precocious maturation and indiscriminate reproduction without compromising the health status of treated animals. From the available literature this study is the first to report the influence of PSM on the blood haematological and biochemical profile of *O. mossambicus*. It is also the first to report on the effect of the PSM on the liver and hepatosomatic index of the fish. Future studies need to focus on the effect of the PSM on liver antioxidant parameters such as lipid peroxidase activity and antioxidant like catalase. The possibility of bioaccumulation of PSM on the liver also needs future consideration.

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Chapter 7

General conclusions and recommendations

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Sex reversal of genetic females to phenotypic males using accessible, and relatively cheap and safe plant sources when fully developed will revolutionize tilapia culture especially among the teeming population of Sub-Saharan Africa which is in dire need of food security. This is premised on the higher growth potential of mono-sex male brood compared to a mix sex culture system. Pawpaw is one of the plant sources that are receiving attention as a possible agent of not only sex reversal but also a reproductive inhibitor to control indiscriminate reproduction that predispose tilapia to overcrowding and stunted growth. Of the different plant components, the seeds contain a variety of compounds that vary in terms of the respective effects of the seed powder, when administered to animals. In Nile tilapia, pawpaw seed meal (PSM) were found to have an antifertility effect, resulting in masculinization of treated fish. This study aimed to establish the optimum inclusion level of the PSM that will result in the highest percentage of male brood, and also the quantity or dose that will inhibit reproductive activities without compromising the overall health of the fish.

Optimum inclusion level of pawpaw seed meal for sex reversal of sexually undifferentiated *Oreochromis mossambicus* fry

Inclusion of PSM in the diet of sexually undifferentiated *Oreochromis mossambicus* fry produced a 77.8% male brood. From an inclusion level of 10 g of PSM/ kg of basal diet, the ratio of male: female was skewed in favour of males. There was progressive increase in the percentage of the male brood as the level of PSM increases up till 20 g of PSM/ kg of basal diet. From 20 to 30 g/kg of the basal diet there was no difference in the percentage of the males, which indicated that 20 g/kg is the optimum inclusion level for achieving the maximum masculinization effect. The results also indicated that the duration of treatment above 30 days were sufficient in ensuring a maximum rate of masculinization, for the percentage masculinization did not differ after 120 days during which the fish received the diets containing similar levels of PSM from that observed when fish received the same treatment diets for only 30 days.

Optimum inclusion level of pawpaw seed meal for reproductive inhibition of pre-vitellogenic *Oreochromis mossambicus*

Inhibition of reproduction in tilapia is employed to either prevent fry production so as to improve growth or to synchronize reproduction thereby having a uniform sized brood. Results of the reproductive inhibition experiment shows that the inclusion level of 30 g/kg of PSM in the diet of pre-vitellogenic (juveniles) was the

<div align="center">137</div>

optimum level for maximum inhibition of reproductive activities in *O. mossambicus*. The endpoint reproductive inhibition indicators in female fish were the most apparently affected by the PSM. The gonadosomatic index (GSI) of the treated females were lower than those of the control and this seems to progress with increase in the level of PSM. The GSI of the treated males on the other hand was comparable with those of the control. Other reproductive parameters such as fecundity and oocytes size (diameter) of the treated fish were also lower than those of the control. These changes increase with increase in the quantity of PSM and the duration of treatment. Apart from the significant reduction in the endpoint indicators of reproductive inhibition, reproductive activities were observed, as indicated by the presence of fry in the culture tanks of the control and low dose groups, but none in that of the high dose groups during the experimental period.

The effect of pawpaw seed meal on the reproductive hormone profile of juvenile *Oreochromis mossambicus*

There was no distinct trend in the plasma level of 11-ketotestosterone established in this study. The implication of this is that either PSM has no or minimal effect on the male reproductive hormone or the quantity included in the diet (maximum; 30 g/kg of basal diet) was not potent enough to elicit any reasonable changes in the 11-ketotestosterone profile. The level of 17β-estradiol among the females declined with the increase in the inclusion level of PSM reaching the lowest among the treatment group that were fed 30 g/kg of basal diet for 60 days, which differed significantly with the control. For the fact that there was no significant difference in the levels of 17β-estradiol of the control with those of the group fed 30 g/kg after 30 days withdrawal of the treatment shows that the reproductive inhibition was reversible up to the 30 g/kg inclusion level.

The effect of pawpaw seed meal on the haematological and serum biochemical parameters of juvenile *Oreochromis mossambicus*

A variation in the levels of haematological parameters is one of the indicators of toxicity of chemical substances in animals including fish. In this study, the PSM did not manifest any toxicity in the form of changes in the levels of the haematological profiles as recorded for the treatment groups. The blood haematological parameters of the treatment groups were comparable with those of the control groups. This shows that at the inclusion of 30 g/kg of basal diet used in this study, PSM is safe for use in tilapia culture. Results of the biochemical parameters obtained in this study indicated that the PSM at an inclusion level of 30 g/kg or less do not affect the levels of the blood cholesterol, total protein, albumin, globulin and glucose concentration in the blood. Since serum biochemical parameters are also indicators of toxicity, these results indicate that the PSM is non-toxic to the fish for the levels used in this study.

Morphological and Histopathological effect of pawpaw seed meal on the liver of *O. mossambicus* juveniles

The liver as an organ whose functions includes detoxification of toxins and metabolites, is prone to damages by these chemical substances. These damages manifest in the form of degeneration, vacuolization or outright necrosis of the liver hepatocytes and parenchyma. The toxic effects can also manifest as changes in the size of liver. In this study, the liver weight and the hepatosomatic index (HSI) of the treated groups were comparable with those of the control, which provided evidence that the PSM did not affect the morphology of the liver. At the micro level on the other hand, there were minor degenerative lesions in the liver hepatocytes of the group fed 30 g PSM/kg of basal diet for 60 days, whereas the group fed the same 30 g PSM/kg of basal diet for 30 days did not manifest these lesions. The fact that the lesions were negligible and also reversible on withdrawal of treatment shows that PSM is safe for use in tilapia culture.

The pawpaw seed meal was well tolerated by the fish and this was evident in the fact that the growth and survival of the fish was not negatively impacted by the PSM. The survival and growth rates of the treated fish in both the sex reversal and reproductive inhibition studies did not differ with those of the control.

In conclusion, provided the concentration are not higher than 30 g/kg, this study shows that using pawpaw seeds as a reproductive inhibitor will not have any negative effect on survival, growth or health status of *O. mossambicus*. This is promising for fish farmers and aquaculture scientists who may now use cost-effective and environmentally friendly pawpaw seed meal for sex reversal and reproductive inhibition instead of expensive exogenous steroid hormones. The same goes for the consumers of tilapia fish, the benefit of eating tilapia fed harmless PSM instead of exogenous hormones cannot be overemphasized.

Recommendations

Due to time and material constraints, the following areas of studies which could not be carried out during the course of this research are hereby suggested for further studies.

Vitellogenin depression in female fish impairs maturation of the eggs since it is the precursor of egg yolk. Conversely, its abnormal presence in male fish (induction of vitellogenesis) is an indicator of endocrine disruption in males. Therefore, there is a need to assay the vitellogenin levels in pawpaw seed exposed *O. mossambicus* to ascertain the effect of the phytochemical on this important hormone which is one of the endpoint indicators of endocrine disruption in fish.

Milt profile such as sperm count, motility morphology and viability are indicators of fertility in males. Therefore, future research on the effect of the phytochemicals contained in the pawpaw seed on these milt parameters will help to elucidate their mode of action in inducing reproductive inhibition in male tilapia, without compromising growth and immune status.

Future research need to consider the possibility of manipulating environmental factors such as temperature, photoperiod and pH to potentiate the action of the PSM in sex reversal so as to produce higher percentage of male brood. Other factors such as rearing density and frequency of administration of the phytochemical deserve further study.

Further research should determine the most suitable method of administration of the phytochemical either through feed or culture water and whether raw seed powder or as extracts (aqueous, ethanol or any other solvent extraction) should be considered as the form in which the phytochemicals need to be included in the diet. Brannan *et al.* (2014) conducted a detailed study characterising the colour, texture, pH, and phytochemical content of 10 pawpaw cultivars. They found that there are several unidentified compounds that the HPLC-PDA-ESI-MS method used to characterise the polyphenolic composition of pawpaw seeds, failed to identify. Future studies should therefore focus on finding the most effective method to identify all polyphenolic compounds in pawpaw seeds, and to link the incidence and level of the respective compounds, to the specific physiological effect, whether it be antioxidant, anti-bacterial or antifertility in nature.

Reference

Brannan RG, Peters T & Talcott ST, 2014. Phytochemical analysis of ten varieties of pawpaw (*Asimina triloba* [L.] Dunal) fruit pulp. *Food Chemistry* 168 (2015): 656-661.

Appendix

Appendix A: 17β-Estradiol assay

17β-estradiol was assayed using Fish Estradiol (E2) ELISA kit (Catalog №: CSB-E13017fh; Lot: C2489421809) manufactured by CUSABIO BIOTECH Co, China

ELISA materials and procedures

Reagents	Quantity
• Assay plate	1(96wells)
• Standard	6 x 0.5 ml
• Antibody	1 x 6 ml
• HRP-conjugate	1 x 6 ml
• Wash Buffer (20 x concentrate)	1 x 15 ml
• Substrate A	1 x 7 ml
• Substrate B	1 x 7 ml
• Stop Solution	1 x 7 ml
• Adhesive Strip (For 96 wells)	4

Other materials

➢ Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 600 nm -630 nm.
➢ An incubator which can provide stable incubation conditions up to 37°C±0.5°C.
➢ Squirt bottle, manifold dispenser, or automated microplate washer.
➢ Absorbent paper for blotting the microtiter plate.
➢ 100 mL and 500 mL graduated cylinders.
➢ Deionized or distilled water.
➢ Pipettes and pipette tips.
➢ Test tubes for dilution.

Assay procedure

Both the standards and samples were done in duplicate according to the manufacturer's instruction, the procedure include;

i. All reagents and samples prepared as directed in the manual
ii. Set a Blank well without any solution.
iii. Add 50µl of Standard or Sample per well. Standard need test in duplicate.

iv. Add 50µl of HRP-conjugate to each well (not to Blank well), then 50µl Antibody to each well. Mix well and then incubate for one hour at 37°C.

v. Aspirate each well and wash, repeating the process two times for a total of three washes. Washed by filling each well with Wash Buffer (200µl) using a multi-channel pipette and let it stand for 10 seconds, complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by decanting. Invert the plate and blot it against clean paper towels.

vi. Add 50µl of Substrate A and 50µl of Substrate B to each well, mix well. Incubate for 15 minutes at 37°C. Keeping the plate away from drafts and other temperature fluctuations in the dark

vii. Add 50µl of Stop Solution to each well, gently tap the plate to ensure thorough mixing.

viii. Determine the optical density of each well within 10 minutes, using a microplate reader set to 450 nm.

Calculation of result

The professional soft "Curve Expert 1.3" was used to make a standard curve

- ❖ Average of the optical density of the blank subtracted from average of the duplicate readings of each standards and samples.
- ❖ Construct a standard curve by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph.
- ❖ The data generated were then linearized by plotting the log of the E2 concentrations versus the log of the optical density and the best fit line determined by regression analysis.

Appendix B: 11-Ketotestosterone assay

The 11-Ketotestosterone hormone was assayed using fish specific 11-Ketotestosterone EIA kit (Item №: 582751; Batch: 0468795) manufactured by Cayman chemical, USA.

Materials and procedures

- 11-Ketotestosterone EIA Antiserum
- 11-Ketotestosterone AChE tracer
- 11-Ketotestosterone EIA standard
- EIA buffer concentrate (10X)
- Wash buffer concentrate (100X)
- Polysorbate 20
- Mouse anti-rabbit IgG coated plate
- 96 well cover sheet
- Ellman's reagent
- EIA tracer dye
- EIA antiserum dye

Assay procedure

Both the standards and samples were done in duplicate according to the manufacturer's instruction, the procedure include;

i.   Add 100µL EIA buffer to non-specific binding wells
ii.   Add 50µL EIA buffer to maximum binding wells
iii.   Add 50µL of appropriate standards to the appropriate well in the plate
iv.   Add 50µL of sample per well. Each sample assayed at two dilutions and in duplicates
v.   Add 50µL 11-Ketotestosterone AChE tracer to each well except the total activity and the blank wells.
vi.   Add 50µL 11-Ketotestosterone EIA Antiserum to each well except the total activity, the non-specific binding and the blank wells.
vii.   Each plate covered with plastic film and incubated for 18 hours at 4°C
viii. Empty the wells and rinse five times with wash buffer
ix.   Add 200µL of Ellman's reagent to each well
x.   Add 5µL of tracer to the total activity wells
xi.   Cover the plate with plastic film and allow the plate to develop in the dark on top the orbital shaker equipped with a cover.
xii.   Read the plate at a wavelength of 412 nm

Calculation of result

- Average the absorbance reading from the non-specific binding wells
- Average the absorbance reading from the maximum binding wells
- Subtract the non-specific binding average from maximum binding average
- Calculate the sample or standard bound/maximum bound for the remaining wells.
- The data is linearized using a logit transformation
- The concentration of each sample is determined by best fit line using regression analysis.

Appendix C: Histology

Automatic tissue processing.
Reagents
1. Alcohol (70 %, 90 %, 95 %, 100 %)
2. Xylene – Sigma-Aldrich
3. Paraffin wax – Merck, Histosec melting point 56 °C

Method
Processing time:
A) Dehydration
1) 70 % alcohol – 1.5 hr
2) 70 % alcohol – 1.5 hr
3) 90 % alcohol – 1.5 hr
4) 95 % alcohol – 1.5 hr
5) 95 % alcohol – 1.5 hr
6) 100 % alcohol – 1.5 hr

7) 100 % alcohol – 1.5 hr
8) 100 % alcohol – 2.0 hr
B) Clearing
9) Xylene – 1.5 hr
10) Xylene – 2.0 hr
C) Impregnation
11) Paraffin wax – 2.0 hr
12) Paraffin wax – 2.0 hr
Thus Total processing time = 20 hr

Appendix D: H&E staining protocol

Reagents
1. 10 % Acid alcohol
10 ml 1 % HCl dissolved in 1 ℓ 70 % alcohol
2. Alcohol (70 %, 95 %, 100 %)
3. Eosin

Stock solution:
10 g Eosin dissolved in 1 ℓ distilled water

Working solution:
10 ml Eosin stock solution dissolved in 90 ml distilled water.
Prepare fresh daily.

For staining:
Add 2 – 3 drops glacial acetic acid per 100 ml before use.
4. Haematoxylin
5 g Harris haematoxylin
100 g Ammonium Alum
50 ml 100 % alcohol
1 ℓ distilled water
2.5 g Mercuric oxide

To prepare: Dissolve haematoxylin in alcohol.
Add Ammonium Alum to distilled water and heat to boiling point.
Immediately add mercuric Oxide and shake until solution has purple-black colour.
Cool rapidly in fridge.
For staining: Filter before use.
Add 4 ml glacial acetic acid per 100 ml of haematoxylin.

5. Scott's tap water
3.5 g NaHCO3
20 g MgSO4
10 ml 37 % Formalin
1 ℓ tap water
To prepare: Dissolve NaHCO3 in tap water first.
Add MgSO4 and formalin.
6. Xylene

144

Method

1. Xylene (10 min)
2. 100 % alcohol (10 dips)
3. 100 % alcohol (10 dips)
4. 95 % alcohol (10 dips)
5. 95 % alcohol (10 dips)
6. 70 % alcohol (10 dips)
7. Rinse in distilled water
8. Haematoxylin (3 min)
9. Rinse in distilled water
10. Rinse in acid alcohol
11. Rinse in distilled water
12. Blue in Scott's tap water
13. Rinse distilled water
14. 2 min in Eosin
15. Rinse in distilled water
16. 70 % alcohol (10 dips)
17. 95 % alcohol (10 dips)
18. 100 % alcohol (10 dips)
19. Xylene (10 dips)
20. Mount with coverslip

Appendix E: Haematological parameters

RBC: Red blood cell counts

HGB: haemoglobin

HCT: Haematocrit (packed cell volume)

 MCV: mean corpuscular volume

MCH: mean corpuscular haemoglobin

MCHC: mean corpuscular haemoglobin concentration

RDW: red cell distribution width

PLT: Platelets (thrombocytes)

WBC: white blood cell counts,

NEU: neutrophils

LYM: lymphocytes

MONO: monocytes

EOS: eosinophils

BASO: basophils