GROWTH, GONADAL DEVELOPMENT, AND BLOOD PROFILE IN AFRICAN CATFISH (*CLARIAS GARIEPINUS*, BURCHELL 1822) FED DIETS CONTAINING COCOA BEAN MEAL

RESEARCH PROJECT REPORT SUBMITTED TO THE DEPARTMENT OF ANIMAL SCIENCE, FACULTY OF AGRICULTURE, UNIVERSITY OF NIGERIA, NSUKKA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTERS DEGREE IN ANIMAL REPRODUCTIVE PHYSIOLOGY.

BY
UZOCHUKWU, IFEANYI EMMANUEL
PG/M.SC/13/64916

SUPERVISOR: PROF. S.O.C UGWU

JULY, 2016

CERTIFICATION

This is to certify that this work was carried out by UZOCHUKWU, IFEANYI EMMANUEL in the Department of Animal Science, Faculty of Agriculture, University of Nigeria, Nsukka. The above statement and the entire project therefore stand approved by:

____
PROF. S.O.C UGWU
(Supervisor)

Dateí í í í í í í í í í

____
PROF. A.G EZEKWE
(Head of Department)

Dateí í í í í í í í í í í í

____
External examiner

Dateí í í í í í í í í í í í í í ..

DEDICATION

I dedicate this report in a special way to My Parents, Mr. and Mrs. David Uzochukwu, my uncle, Chief. Ufondu, B.O, and to my younger sister Mrs. Orji-Uzochukwu C.B. for the hope they had in me even when things seemed rough.

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Abstract

A 63 day experiment was conducted to determine the effect of varying dietary levels of cocoa bean meal (CBM) on the body growth, gonadal development, and blood lipid profile of Clarias gariepinus catfish. Four hundred C. gariepinus post-fingerlings were randomly assigned to five treatments with eighty fish per treatment. Each treatment was replicated four times with 20 fish per replicate in a randomized completely block design (RCBD) with sex as a block. Diets (35% crude protein) containing 0, 10, 20, 40, and 50% CBM were formulated to be fed to the fish in treatments 1, 2, 3, 4, and 5 respectively. Before the commencement of feeding the experimental diet, ten fish (five males and five females) were randomly collected from the various treatments, weighed, dissected, and the gonads (ovaries and testes) harvested and fixed in a bouins fluid. The fixed samples were them taken to the laboratory for histologic and histomorphometric studies. After three weeks of feeding the experimental diets, eight fish (four males and four females) were randomly selected from the various treatments. The gonads were also harvested and fixed for laboratory studies. Further random selections of the fish for laboratory studies were done on fortnightly basis till the 9th week of the study. At the end of the study, four fish from each treatment were selected and blood samples were collected from them for haematology and blood lipid profile. Body weights and lengths measurements were made and recorded on weekly basis. Feed was given to the fish at 5% bio mass and the water changed weekly to ensure freshness. Results of the study showed that the average final standard length, average standard length gain, average final total length, and total length gain were not significantly ($P \geq 0.05$) different among the treatments. The average total weight and total weight gain however differed significantly ($P < 0.05$) among the treatments.T2 (10% CBM) had the highest average weight gain value (8.44g). T1 (control) had value (3.59g) which was significantly ($P < 0.05$) lower than T2. T5 (50% CBM) has the lowest value (0.98g) which was also significantly ($P < 0.05$) lower than T2 and the control. T3 and T4 had values (5.55, and 3.59g respectively) which did not differ significantly ($P \geq 0.05$) from those of T2 and the control. Also, ovarian weight, testicular weight and GSI values were shown to be significantly ($P < 0.05$) different among the treatment groups. T2 females had the highest ovarian weight and GSI values (2.356g and 6.22) which significantly ($P < 0.05$) different from T3 (0.58g and 1.65 respectively), T1 (0.358g and 1.124 respectively), and T5 (0.36g and 1.21 respectively) which had the least values. T4 had values (1.27g and 3.95 respectively) which did not differ significantly ($P \geq 0.05$) from the other groups. Similar result was obtained for the males. T2 and T4 had the highest testicular weight and GSI values. The least values were reported for T5. T3 and the control were found not to differ significantly ($P < 0.05$) from other groups. The results obtained for the histology and Histomorphology showed that the gonads of fish in T2 and T4 were better developed than those of the other groups. T5 was least developed as indicated by lower GSI, tubular diameter and oocyte stages and diameters. Results of the haematology showed there were no significant ($P \geq 0.05$) different in the haematological indices of Packed cell volume, haemoglobin, red blood cells, monocyte, eosinophil, and basophil. However, the white blood cell count, neutrophil, and lymphocyte were significantly ($P < 0.05$) different among the treatment groups. The blood lipid profile showed that low density lipoprotein, total cholesterol, and triglyceride differed significantly ($P < 0.05$) among the treatment groups. High density lipoprotein was however not significantly ($P \geq 0.05$) different. From the result, it was therefore concluded that feeding cocoa bean meal up to 40% dietary inclusion improved growth and gonadal development. Above this level however (i.e. at 50%), the fish were adversely affected. 20 and 40% CBM dietary inclusion is hence recommended if improved reproductive development and performance is of interest.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of study

It has long been recognized that shortage of animal protein is one of the limiting factors in attainment of food security in Nigeria. It is also established that the minimal protein requirement of an individual is 75g/person/day of which 40g should be from animal source (Federal Ministry of Agriculture and Rural Development: FMARD, 2011). According to FAO (2014) current animal protein consumption in Nigeria is given at 7g/person/day indicating a less than 16% contribution of animal protein to the cumulative protein consumption. The situation worsens with the ever increasing population of the country (FMARD, 2011) leaving a bleak future for human nutrition in Nigeria if animal protein sources should be left to conventional farm animals alone.

Of all the sources of animal proteins, fish is the cheapest and these fishes are abundantly found in nearly all aquatic environments spawning up to 31,900 species making it the largest of all classes of vertebrates in species diversity (Oyinlola, 2011). Due to poor supply and high cost of animal protein in Nigeria (Ampofo-Yeboah, 2013), fish consumption has been reported to constitutes about 41% of the total animal protein intake and about 22% of the total protein intake (FAO, 2011). According to FAO (2012), fish provides more than 1.5 billion people with almost 20 percent of their average per capita animal protein intake, and 3.0 billion people with at least 15% of such protein. Fish farming has grown strongly as an effective way of generating food and income from dwindling land space, as fish supplies from open water and lagoons continue to fall and human population increases (Adebayo and Adesoji, 2008). Despite domestic supply from both capture and culture fisheries of less than 0.7 million metric tons per annum in Nigeria, about 0.7 million metric tons of fish is imported annually to augment the dietary requirement of its citizens, making Nigeria the largest importer of frozen fish in Africa (FMARD, 2011). This as stated by the Federal department of Fisheries (FDF) (2007) to create huge foreign exchange demands on the economy. Fish and fisheries products are essential component of the economic and social health wellbeing of many countries and societies, and aquaculture has been identified as the next viable solution in reducing importation (Oyinlola, 2011; Pepple and Ofor, 2011).

African catfish (*Clarias gariepinus*; Burchell, 1822) is a major aquaculture species in the list of culturable species which has gained widespread recognition as a promising species in

aquaculture production in Nigeria (Taiwo, 2008; Lenient *et al*., 2008). It is an economically important food fish, cultured primarily in freshwater ponds in tropical countries (Babalola and Apata, 2006). The reasons for the preferential use of catfish in Nigeria include its omnivorous feeding manner; fast growth rate; high yield potential; high fecundity; air-breathing characteristics; good market potentials; and high degree of tolerance to diseases, as well as harsh and poor environmental conditions; (Ayinla *et al*., 1994; Rad *et al*., 2003; Anyanwu *et al*., 2007).

In artificial breeding of fish, efforts are channeled towards obtaining breeding stock with quality sperm and ripe ovary for the production of large number of good quality fries (Oyinlola, 2011). Although recent efforts have been made in increasing the multiplication and supply of seeds (as seen in the catfish seed multiplication project of West Africa Agricultural Productivity Programme: WAAPP), it is still faced with several challenges. Estimates by the United Nations development project (UNDP) assisted base line study gave the annual fingerlings requirement for Nigeria to be 250 million while domestic supply stood at 7.2 million (Nwokoye *et al*., 2007). Among the factors affecting fish seed quality as stated by Conyurt and Akhan (2008) and Dada and Ebhodaghie (2011) are genetic; broodstock fertility, nutrition (quality and quantity) and outcomes of modern agricultural practices which introduce chemical residues (fertilizers and insecticides) into natural water sources used for culture. Also of importance are the negative effects of common hatchery practices like transportation, cleaning, handling, and use of chemicals, poor water quality and crowding of fishes which are stressors on reproduction (Billard *et al.,* 1995). These factors affect fertilization in artificial reproduction resulting in the production of low quality fish seeds and thus prompting research into various ways of mitigating their effects and producing high quality fish seeds (Oyinlola, 2011). In order to improve culture techniques, it is extremely important to understand the mechanism linking gonad development and reproductive performance (Cek and Yilmaz, 2007). According to Saka *et al.* (2015), histology is an important tool used in assessing fish reproductive health and to show the initial signs of lesions or alterations not easily identifiable during the macroscopic examination of fish tissue. Many researchers (Schulz *et al*., 1997; Yalcin *et al*., 2001; Cek and Yilmaz, 2007; Saka and Adeyemo, 2015; Saka *et al*., 2015) have greatly studied the gonadal development in *C. gariepinus* and their maturation. This has given rise to an in-depth exposé on the reproductive biology of this species. Attempts have been made to improve growth and maturation in order to increase productivity in catfish seed production. The continuing expansion of aquaculture and

perhaps the advocates of organic farming and minimal use of chemicals in crop and animal production, because of their implications, for man has engineered a shift from synthetic drugs and chemical to natural plants (neutraceutical) to aid artificial breeding in fishes (Dada and Ajilore, 2009). Hence medicinal plant/phytochemicals which were then little thought of are now researched, evaluated and developed into additives or drugs with little or no side effects (Adedeji *et al*, 2006). Medicinal plants and other plant products and by-products are now being used as fertility enhancers in production of *Clarias gariepinus* spp. (Oyinlola, 2011). Gonadal development in all species of animals is influenced by gonadotropic hormones produced as a response of hypothalamus to environmental cues such as photoperiods, feeding and temperature (Al-Khamees, 2009). Ugwu and Ugwuoke (2013) from their research reported that some of these plants which are known to contain phytoestrogens have more pronounced effect on reproductive structures than on other body tissues.

Cocoa powder is a product from the beans of tropical plant, *Theobroma cacao* which is mostly found in West Africa, South and Central America and tropical areas of Asia (Weisburger, 2001). Cocoa and its products (cocoa butter, cocoa powder, dark chocolate, and cocoa liquor) are known to be therapeutic because of their content of important substances as antioxidants (mainly flavonoids, epicatechin, catechin, and procyanids), methylxanthine (caffeine and theobromine), nitrogenous compounds and minerals (Adamson *et al.,* 1999; Visioli *et al*., 2000; Greer *et al.,* 2001; Rios *et al.,* 2003; Kelm *et al.,* 2006; Tomas-Barberan *et al.,* 2007; Maleyki and Ismail, 2008; Latif, 2013). Polyphenols (flavonoids) are reported to exhibit multiple biological effects including; anti-oxidant, antiviral, antibacterial, anti-inflammatory, vasodilatory, anticancer, and anti-ischemic in vitro (Procházková *et al.*, 2011). The phenolic agents in cocoa confer it with its resistance attribute to peroxidation in addition to antioxidant and immune regulatory effect (Visioli *et al*., 2000). In addition, flavonoids are known to have the ability to modulate cytochrome P- 450 activity. Flavonoids and procyanidin exert their effects (both in-vitro and in-vivo) as antioxidants, free radical scavengers and chelators of divalent cations (Adamson *et al.,* 1999; Vinson *et al.,* 1999; Ampofo-Yeboah, 2013). Diets rich in antioxidants have been reported to reduce the damage free radicals can have on sperm and biological functions, hence chocolate has been recommended for males with fertility issues (www.fertilityassociates.co.nz/boosting-your-fertility.aspx; Yildirim *et al*., 2014). The antioxidant products found in cocoa have been reported to inhibit the oxidation of low density lipoprotein (LDL-cholesterol); thus, they show a protective effect against heart disease (Weisburger, 2001).

Plant products such as *Garcinia kola* seed have been reported to improve growth performance in catfish broodstock (Dada and Ikuorowo, 2009). On the other hand, polyphenols including flavonoids, isoflavones, lignins, coumestanes, which are all abundant in plants and plant by-products are phytoestrogens (functionally similar to natural androgens and estrogens) and have been reported to function as endocrine disrupting chemicals (EDC) which exhibits antifertility and abortifacient properties (Ampofo-Yeboah, 2013). Histological studies on fish have shown that some plant products. Pawpaw seeds (Ekanem and Okoronkwo, 2003; Jegede and Fagbenro, 2008), and Moringa oleifera (Bose, 2007) appear to be an effective sterility inducing agent through the disintegration of gonadal cells, preventing testicular and ovarian development into spermatids and oocytes. However, Hostetler *et al*. (1990) reported no treatment effect on fertility in rats when cocoa powder supplemented diet is fed.

Factors like genetic, nutrition, temperature, photoperiodicity, confinement, handling, use of chemicals, poor water quality and stocking density impose stress on fishes and thus affect their growth and reproductive performance.

1.2 OBJECTIVES OF THE STUDY

The broad objective of this study was to determine the effect of feeding diets supplemented with cocoa bean cake (meal) on body growth, gonadal development, haematology and blood lipid profile of *C. gariepinus*.

The specific objectives are to determine

- The effect of dietary inclusion of cocoa bean meal on the growth performance of *C. gariepinus* catfish.
- The effect of dietary inclusion of cocoa bean meal on the gonadal development of *C. gariepinus* catfish.
- The effect of dietary inclusion of cocoa bean meal on the blood haematology of *C. gariepinus* catfish.
- The effect of dietary inclusion of cocoa bean meal on the blood lipid profile of *C. gariepinus* catfish.

1.3 JUSTIFICATION OF RESEARCH

The demand and production of fresh fish (especially cultured fish products) have been on the increase particularly with the efforts of the federal government to cut down on importation to

increase foreign exchange earnings. This has given rise to a need for suitable methods of fish seeds production to meet the high demand edible fish (Dada and Ebhodaghe, 2011). On this note it has become extremely important to understand the relationship between gonad development and reproductive performance of fish in order to improve culture techniques. (Cek and Yilmaz, 2007).

The tilting away from the use of synthetic chemicals in animal agriculture in recent times has necessitated research into the use of natural substances in curtailing the negative impacts of these chemical stressors on fish reproduction and hence improving efficiency of artificial fish breeding. The case of poor survival of catfish larvae and frys have been associated with poor quality eggs produced by the females which is more common than male fertility issues. Also coupled with the fact that there is no standard diet for broodstocks or young juveniles reared for breeding purposes, there is need to use plants with antioxidant and medicinal properties to enhance the performance and fertility of broodstocks (Dada and Ebhodaghe, 2011). Cocoa bean meal, although in competitive demand between man and animals and thus somewhat costly when compared to other non-conventional feedstuffs can go a long way being beneficial as it is readily available and will be easy to adopt. Also the extra incurable cost from its use will be made up for by improved reproductive performances. This study therefore seeks to key into this peculiar area of using õneutraceuticalsö to improve productive and reproductive development and thus recommend strategies to hatchery operators.

CHAPTER TWO

LITERATURE REVIEW

2.1 AQUACULTURE PRODUCTION: NIGERIA

Aquaculture involves the cultivation of fresh and salt water organisms in a diverse aquatic environment that include freshwater, brackish, and marine systems where all its life processes can be controlled (Engle and Stone, 2005). It can be contrasted with commercial fishing, which is the harvesting of fish and other seafood for commercial profits. Aquaculture production occurs in ponds, raceways, cages, rafts, baskets, lines, recirculating systems, and by ocean and reservoir ranching. Its products are farmed for food consumption, for sport fishing, for bait, for clothing (alligator skins), for pets (Ornamental fish and feeders), and for industrial processes (seaweeds for agar and carrageenan) (Stickney, 2000). Aquaculture is an important venture for the economic development of a country. It is important in the diversification of the economy especially in Nigeria where it is purely oil based in Nigeria; a potential means for achieving food security; generation of employment; savings and generation of foreign exchange through export of fish and fish products (Ekelemu, 2012). Aquaculture is also important source of animal protein for human population, contributing more than 60% to the world protein supply especially in developing countries (FAO, 2007; Oyakilomen and Zibah, 2013). More than 210 species of finfish, crustaceans, mollusks, and aquatic plants are reared worldwide in various aquaculture projects with about 99% grown for human consumption (Engle and Stone, 2005). In Nigeria, the most important of these species include: prawn, catfish and tilapia. And according to Engle and Stone (2005), Species that are good aquaculture candidates should be: easy to reproduce in a controlled environment; accept prepared feeds; are hardy; tolerate a wide range of water quality conditions; and have high market value.

Aquaculture was developed over 2000 years ago (900 B.C.) in countries like China, Italy, and Egypt; with Asia (e.g. China having the highest production output in the continent) and America presently leading in the global production (Ozigbo *et al*., 2014). With the exception of Chile, the top 10 countries in world aquaculture production are all Asian countries and most of them (with the exception of Japan) are lesser-developed nations (FAO, 2002; Engle and Stone, 2005). According to Ayinla (2012), aquaculture is the fastest growing food producing industry in the world with its global production having quadrupled over the past twenty years and is likely to

double in the next fifteen years consequent upon the continuing increase in world demand for cultured fish and wild fisheries approaching their biological limits. Aquaculture activities in Nigeria started in early 1940øs (about 50 years ago) when the colonial administration established a fisheries organization in Lagos during World War II (Olagunju *et al*., 2007). This was followed by series of aquaculture researches and developmental programmes which have been driven by social and economic objectives. Attempts to reduce the major constraints to the rapid development of aquaculture in Nigeria featured prominently in the second phase of the National Development Plan (Ezenwa, 1994). Extension activities in the 1960øs and 70øs given by the government demonstration fish farms at Itu and Opobo boosted the awareness process and adoption of the technologies during the early days of aquaculture development in Nigeria (Ajenifuja, 1998). The then enthusiasm gave rise to a significant individual participation in fish-farming within the Niger Delta Region of Nigeria (Inyang, 2001). In 2009 according to statistics, Nigeria became the major aquaculture producers in Africa with a production output of about 15,489 tonnes per annum followed by Egypt, Zambia, Madagascar, Togo, Kenya, and Sudan in a decreasing order (Ekunwe and Emokaro, 2009). Nigeria, with over 160 million people, a land mass of 923, 766km^2 and a coastline measuring approximately 853 kilometers, has about 1.75 million hectares of suitable sites for aquaculture development. According Ekelemu (2012), as at 2012 the domestic aquaculture production was about 620,000 tonnes with an importation of 700,000 metric tonnes (a total supply of 1.32 million metric tonnes). The annual demand was placed at about 2.66 million tonnes which leaves a large demand-supply gap (Ekelemu, 2012). Nigeria spends N100 billion on fish importation annually which means that more than USD 600 million are spent in hard currency and thousands of jobs created for these foreign countries instead of locally (USAID, 2010; Oota, 2012). According to Akinrontimi *et al*. (2011), the supply of fish in Nigeria is mainly from four sources which include: artisan fisheries, industrial trawlers, aquaculture and imported frozen fish. Nwiro (2012) described the fish farming industry as being in the infant stage when compared to its local large market potentials. Table 1 shows the projected human population, fish demand and supply in Nigeria from the period of 2000 to 2015.

Table 1. Projected Human Population, Fish Demand and Supply in Nigeria (2000–2015)

Year	projected population (Million)	Projected fish demand (Tonnes)	Projected domestic fish supply (Tonnes)	Deficit (Tonnes)
2000	114.4	1,430	467.098	962.902
2001	117.6	1,470	480.163	984.836
2002	121.0	1,412	507.928	1,004.572
2003	124.4	1,555	522.627	1,063.082
2004	128.0	1,600	536.917	1,063.072
2005	131.5	1,643	552.433	1,091.317
2006	135.3	1,691	567.948	1,023.301
2007	139.1	1,732	583.872	1,154.873
2008	143.0	1,782	600.612	1,186.887
2009	147.1	1,838	617.353	1,221.397
2010	151.2	1,810	634.500	1,255.440
2011	155.5	1,943	652.606	1,291.143
2012	160.0	2,000	689.958	1,328.508
2013	164.0	2,113	709.683	1,365.042
2014	169.1	2,175	730.248	1,404.067
2015	174.0	2,055	671.492	1,444.752

Source: (FDF, 2008) (Tonnes × 1000)

2.2: CATFISH: *Clarias* species

Catfishes represent a diverse group of ray finned fish of various sizes and behaviour, named after their prominent barbels, which resemble a catøs whiskers. They are widely distributed in Africa and Asia (Ali, 2015). Many of these species are nocturnal while others are diurnal (Wong, 2001; animal-world.com). Catfishes have inhabited all the continents at one time or the other and are most diverse in tropical South America, Asia and Africa. Only one family is native to North America and Europe (Nelson, 2006; Lundberg and Friel, 2003). More than half of the world catfish species live in the Americas and they are known by different unstandardized names like: õmud catö, õpolliwogsö, or õchuckleheadsö (http://www.tpwd.state.tx.us). Catfishes are found in

a range of diverse habitat starting from the freshwater; shallow running water; underground (hypogean); caves (troglobitic); and salt water (Langecker and Longley, 1993; Bruton, 1996; Hendrickson *et al*., 2001; Schafer, 2005; Monks, 2006; Froese and Pauly, 2007). The main cultured species of this group include: *Clarias gariepinus, Clarias batrachus, Clarias macrocephalus,* and *Clarias aguillaris* (Ali, 2015). In Africa, there more 100 different species of the genus *Clarias* have been described. A more recent anatomical and biographical studies by Teugels (1982a, 1982b, 1984) have however recognized 32 valid species. The large African species which are important for aquaculture belongs to the subgenus *Clarias* and five main species have been recognized in earlier systematic study by David (1935) which include*: Clarias anguillarus; Clarias senegalensis; Clarias lazera; Clarias mossambicus; and Clarias gariepinus*.

2.2.1 AFRICAN CATFISH (*Clarias gariepinus*)

The *Clarias gariepinus* or the African sharptooth catfish of the family clariidae (the air breathing catfishes) (https://en.wikipedia.org), is a freshwater eurytopic species (Ali, 2015) and represent the most commonly cultivated fish in Nigeria. The African sharptooth catfish is a large, eel-like fish, usually of dark gray or black coloration on the back, fading to a white belly. The superior performance of the *C. gariepinus* when compared with to other *Clarias* species has resulted to their wide introduction to areas outside their habitat from the temperate to tropics (Verreth *et al*., 1993). It has been referred to as an opportunistic omnivore, varying its feeding according to availability (Uys, 1989). This catfish has been reported as being second in size only to the vundu of the Zambesian waters in Africa (www.ecotravel.co.za), although Fish statistics base suggests the African sharptooth catfish surpasses this species (vundu) in both maximum length and weight (Rainer and Daniel, 2014). According to Adewumi and Olaleye (2011), the favoured catfish species in Nigeria aquaculture include: *C. gariepinus, Heterobranchus bidorsalis, Clarias gariepinus x Heterobranchus bidorsalis hybrid (HeteroClarias) and Clarias nigrodigitatus.* The zoological classification and basic anatomical characteristics of the *Clarias gariepinus* is shown in table 2.

Table 2: Zoological classification of *Clarias gariepinus* (Burchel 1822)

Kingdom	Animalia
Phylum	Chordate
Class	Actinopterygii
Order	Siluriformes
Family	Clariidae
Genus	*Clarias*
Species	*C. gariepinus*
Binominal name:	
Clarias gariepinus (burchell, 1822)	

Source: https://en.wikipedia.org/wiki/*Clarias_gariepinus*

2.2.2 Catfish Production in Nigeria

The story of aquaculture in Nigeria is essentially the story of catfish culture and the hope of fish supply in Nigeria hangs on its development and culture (Adewumi and Olaleye, 2010). The first attempt at Catfish farming was in 1951 at a small experimental Station in Onikan, Lagos, Nigeria (Longhurst, 1961). Anetekhai, *et al*. (2004) reported that catfish farming in Nigeria started in 1951 with the construction of Panyam Catfish Farm located in the present day Plateau State in the North Central part of Nigeria. What is discernible in these views according to Anetekhai (2013) was that aquaculture started in different parts of the Country about the same period. Olaniyan (1961) and Ajayi (1971) pointed out that Panyam (Northern Nigeria) experience was based on the use of *Cyprinus carpio*, while Onikan experimental fish trials were conducted using Tilapia species. At the onset (1951-1971), Catfish Farming was dominated by Government and its agencies (State and Federal) with direct involvement in Catfish production. The developmental progress in catfish production was facilitated by series of research, by government programmes and projects which have been greatly supported and funded by international organizations like the World Bank, FAO and UNDP amidst agencies, institutes, organizations and ministries which are directly or indirectly involved in catfish production and extension to farmers. As stated by Anetekhai (2013), involvement of the private sector in catfish production started during the period 1981 to 1991 and by 1991 to 2001, it became clear that catfish production must be seen and managed as a business if substantial progress is to be

achieved. The state of catfish production today is such that government provides enabling environment (development of roads, signing of international agreements), while the private sector is charged with commercial production (Anetekhai, 2013). This is evident in the catfish seed multiplication, distribution and production assistances commissioned and sponsored in several locations in Nigeria by World Bank through the West African Agricultural productivity programme in 2012.

The development and hope of aquaculture in Nigeria hangs on the development and culture of African catfish especially as the fish stock in the wild have approached and exceeded the point of maximum sustained yield (Adewumi and Olaleye, 2011). African catfish, as stated by Hogendoorn (1983) is a suitable alternative to tilapia in subsistence fish farming in Africa and using low grade feed composed of some local agricultural by-products; the yields of catfish from ponds could be as much as 2.5 times higher than those of tilapia. The *clariidae* family of catfish are the most commonly cultivated fish in Nigeria due to attributes which according to Wikipedia (https://en.wikipedia.org) include:

- Fast growth rate and ability to feed on a large variety of agricultural by products
- It is hardy and tolerates adverse water quality conditions
- It can be raised in high densities, resulting in high net yields (6616 t/ha/year).
- In most countries, it fetches a higher price than tilapia, as it can be sold live at the market
- It matures fast and is relatively easy to reproduce in captivity

- And other general benefits of aquaculture like: income generation for the farmers, job creation (as seen in Table 3), foreign exchange earnings, and dietary protein supply.

Table 3: Estimated Catfish production (metric tonnes) and job creation from catfish in Nigeria (2001 -2012)

Species	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
C. gariepinus	19,518	24,530	24,542	35,160	45,084	67,662	68,100	15,000	137,516	180,482	199,015	228,508
***Clarias* hybrid**	390	491	491	703	902	1,353	1,362	2,300	9,168	12,032	13,268	15,234
Heterob. Spp	1,464	1,840	1,841	2,637	3,381	5,075	5,108	8,625	6,112	8,021	8,845	10,156
Total	21,372	26,861	26,874	38,500	49,367	74,090	74,570	125,925	152,796	200,535	221,128	253,898
Total job	21,372	26,861	26,874	38,500	49,367	74,090	74,570	125,925	152,796	200,535	221,128	253,898

Source: Anetekhai (2013); Total job: Total job creation-production and marketing; Heterob. Spp: Heterobranchus spp.; *C. gariepinus*: *Clarias gariepinus* spp.

While catfish production (and aquaculture as a whole) is gaining popularity and momentum in Nigeria, the sector is still underdeveloped and inefficient. This has been attributed to the importation and resultant high cost of farming inputs such as fish meal, fish feed, fish and health supplies. Poor management; high cost of construction and purchase of ponds; faulty data collection; lack of environmental impact consideration; and inadequate marketing strategies for the products are also important factors (Adewumi and Olaleye, 2011). Locallly produced fingerlings are also expensive and many are of questionable quality (Babatunde, 2014). The estimated catfish production from aquaculture between 2001 and 2012 are presented in Table 3.

2.3 NUTRITIONAL REQUIREMENTS OF CATFISH

Research on the nutritional requirement of African catfish, *Clarias gariepinus* (Burchell, 1822), was mainly initiated in the Netherlands, S. Africa and Israel (Hogendoorn *et al*., 1983; Henken *et al*., 1986; Machiels and Henken, 1986; Uys, 1989; Degani *et al*., 1989). The objective of feeding fish is to provide the nutritional requirements for optimum growth, good health, optimum yield and minimum waste within reasonable costs so as to optimize profits (Schmittou *et al*., 1998). Diets are meant to supply all the essential nutrients and the energy for all the vital physiological functions such as growth, reproduction, and the maintenance of normal health condition (Jobling, 1995; Ugwu and Ugwuoke, 2013). According to Ugwu and Ugwuoke (2013), the major issues in aquaculture include ensuring the quality of the end product (flesh) and stock replacement (reproductive performance) both of which are highly influenced by nutrition. Feed accounts for a major part of the total operations of an average fish farm and the resultant performance of fish depends on the feed quality as well as the feeding management (Ali, 2001).

Potential feedstuffs come in many shapes and sizes, and different fish species have become adapted to utilize many different food types at different stages of their life ranging from other fish species, to water plants, and even hippopotamus dung (Jobling, 1995). Given the euryphagic nature of the species (Bruto, 1979), it is not surprising that it is able to efficiently utilize a wide variety of ingredients (Fagbenro *et al*., 2003) given rise to its consideration in some quarters as an ideal õbio waste management instrumentö (Sambhu, 2004). In Africa, the high cost of formulated commercial fish feeds is a major constraint to the expansion and growth of the aquaculture sector (Hecht, 2007), and this has prompted a concerted effort, particularly in

Nigeria, to seek for alternative non-conventional feed ingredient to reduce cost and also enhance productivity (FAO, 2015) . However, this search requires a sound knowledge of the availability of the various feedstuffs in the environment, their digestibility and cost (Ali, 2001).

Qualitatively, there are 40 nutrients identified for the normal metabolic function of catfish while their quantitative requirements have also been identified (Robinson *et al.*, 2001). The established nutritional requirements for catfish have generally been based on weight gain and feed efficiency of small fish raised under laboratory conditions presumed to be near optimum. The major classes of nutrient for catfish include energy (carbohydrates and lipids), proteins, vitamins and minerals.

2.3.1 Energy Requirements

The performance of any activities, such as swimming, mating, and fight requires the expenditure of energy and many feeding standards are based on energy needs. The energy sources in the food are organic materials, consisting of carbohydrate, lipids, and proteins. These organic molecules consist mostly of carbon, hydrogen, and oxygen in varying proportions with minor quantities of nitrogen, sulphur, phosphorous, and other elements (Jobling, 1995). Although catfish feed intake may not be strictly regulated by dietary energy, balance of dietary energy in relation to other dietary nutrients is important when formulating catfish feeds (Robinson *et al.*, 2001). This is important because when non-protein energy is deficient in the diet, the more expensive protein will be used for energy. On the other hand, when the dietary energy content is too high, catfish may not eat as much as expected, resulting in too low intake of essential nutrients. Excessively high dietary energy can lead to visceral and tissue fat and reduce dress yield and consequently reducing shelf life of frozen products (Robinson *et al.*, 2001).

Although, the absolute energy requirements for catfish are unknown, estimates have been made by measuring weight gain or protein gain of catfish fed diets with known content of energy (Hossain *et al.*, 1998). Energy requirements for catfish have generally been expressed as a ratio of digestible energy (DE) to crude protein (DE/P), and it ranges from 31.0 to 50.2 kJ g^{-1} with a DE/P of 35.6 to 39.8 kJ g^{-1} being adequate for use in commercial catfish feeds (Phonekhampheng, 2008). Increasing the DE/P ratios of catfish diets above this range will increase fat deposition and reduce processed yield, and in contrast if the energy value is too low the fish will grow slowly (Nematipour *et al.*, 1992a). According to ADCP (1983), best growth rates and feed conversion ratio are achieved when catfish are fed diets containing 35-42% crude protein and a calculated digestible energy of 12Kj g^{-1}. According to FAO (2015), the Gross

energy and digestible energy requirements are around 19 kJ/kg and 14 kJ/kg, respectively, with an average protein to energy ratio of 27 mg/kJ. The energy requirement for the different catfish ages include: 3.0 ó 4.0 kcal/g (fry and fingerlings), 2.5 ó 3.5 kcal/g (growers) and 3.0 ó 4.0 kcal/g (brood stock) (FAO, 2015). However, energy requirements are not uniformly expressed and this hampers objective comparison and the deduction of the appropriate energy requirement. Different authors have used the gross energy (Degani *et al*., 1989; Khan and Jafri, 1990), the metabolisable energy (Fagbenro, 1992; Hassan *et al*., 1995) and the digestible energy (Jantrarotai *et al*., 1996; Samantaray and Mohanty, 1997) estimates in formulating their diets.

2.3.2 Dietary Carbohydrate

Carbohydrates are a group of compounds composed of carbon, hydrogen, and oxygen and are the basic form of energy stored in the seeds, roots, and tubers of plants. According to Robbinson *et al*. (2001), the animal blood contains about 0.05% to 0.1% circulating glucose, which is used for energy and is replaced from the stored glycogen in the liver and muscles. Apart from their role as energy source, carbohydrates also serve as tissue constituents and precursors of some metabolic intermediate. It has been reported that since animals are capable of synthesizing carbohydrates from proteins and lipids, they do not necessarily require dietary carbohydrate for growth and functions (Robbinson *et al*., 2001). Teleosts generally have a limited capacity to assimilate and metabolize dietary carbohydrates (Cowey and Cho, 1993), although there is some controversy about the ability of *C. gariepinus* to utilise dietary carbohydrates efficiently (Ali, 2001).

Between 15 and 32% (average of 27%) of soluble carbohydrate are recommended for catfish in addition to about 3 ó 6 % carbohydrate present as crude fiber (mainly cellulose) for growers and a maximum of 21% for larvae and early juveniles (FAO, 2015). Ali (2001) suggests that *C. gariepinus* cannot utilize dietary carbohydrate levels above 35 %. On the other hand, it was found that dietary carbohydrate levels of between 26 and 32 % had a significant protein sparing effect (Pantazis, 2005). Thus, the greater use of carbohydrates in catfish diet formulation was advocated. Although carbohydrates are sources of cheap energies, they are also important in the manufacture of the feed. The starch content binds the feed together when heated while expanding the feed so that they become water-stable and can easily float. As stated by Hogendoorn *et al*. (1983) catfish feed usually contains 25 % or more soluble carbohydrates plus 3 to 6 % less soluble carbohydrates that are in general present as crude fiber. African catfish cannot digest crude fiber well, so dietary fiber level should be kept as low as possible this is largely considered

by commercial catfish feed producers who do not include up to 5% crude fiber in feeds (Phonekhampheng, 2008).

2.3.3 Dietary Lipids

Lipids are highly digestible source of concentrated energy; it contains about 2.25 times as much energy as does an equivalent amount of carbohydrate serving as energy stores (Craig, 2009). Among its physiological roles in metabolism include protein sparing, supplying essential fatty acids, a means of absorption of fat-soluble vitamins, and precursors for the steroid hormones and other compounds. Although the use of lipids in the fish diet may increase palatability (and possibly feed intake), the body lipid stores also affect the flavor of fish in addition to maintaining neutral buoyancy (Robbinson *et al*., 2001). According to Robbinson and Li (2001), the type and amount of lipids (vegetable and animal lipids) used for the diets is dependent on the essential fatty acid requirements, cost considerations, and quality of fish flesh desired.

Essential fatty acids (EFA) are those that must be provided in the diet because they cannot be synthesized in the animaløs body. Catfish appear to have the ability to synthesize most of their fatty acids; thus, nutritionally there may be no õbestö level of dietary lipid except that needed to provide EFA. Generally, weight gain and feed efficiency are depressed when fish are fed diets containing 15% or more lipid. Since lipid is a concentrated source of energy and can spare the more expensive protein, some quantities of lipid should be included in catfish diets. Energy that is not utilised immediately is stored for future use as glycogen and carcass fat. Since glycogen reserves in fish are usually low, the main energy stored is fat (Ali, 2015). Experiments show that during starvation or food restriction in fish, most of the metabolic energy is derived from lipid and, to a more limited degree, protein and carbohydrate (De Silva and Anderson, 1995).
However, when in excess, dietary lipid may result in excessive deposition of fat in the body cavity, visceral, and tissues that may adversely affect processing yield, product quality, and storage of processed products. Also, high-lipid feeds are more difficult to pellet, but if needed, supplemental lipid can be sprayed onto the finished feed pellets. Lipid levels in commercial feeds for catfish rarely exceed 5ó6%. About 3ó4% of the lipid is inherent in the feed ingredients, with the remaining 1ó2% being sprayed onto the finished pellets (Robbinson and Li, 2005). Palm oil has been added in the diet of catfish at 2.5% to frys (Davies and Ezenwa, 2010) and at 1%

inclusion for fingerlings (Anyanwu *et al*., 2011) without any adverse effect. The source of the dietary lipids also appears not to affect growth and performance of catfish (Solomon *et al*., 2012). Spraying feed pellets with lipid increases dietary energy and aids in the reduction of feed dust (õfinesö).

2.3.4 Protein and Amino Acids Requirements

Proteins are organic compounds composed of organic acids and comprises about 70% of the dry weight of fish muscle (Wilson and Halver, 1986; Ali, 2001). It is composed of amino acids and varies in quality depending on the source of the protein (whether plant or animal origin). After ingestion, proteins undergo hydrolysis to release amino acids that may be used for synthesis of tissue proteins or, if in excess, used for energy (Robinson *et al*., 2001). According to Cowey (1995) a considerable proportion of dietary amino acids are not used by fish for anabolic processes, but are catabolised as an energy source. A dietary supply of protein is needed throughout life for maintenance and growth of catfish, since protein is in a dynamic state, continually being synthesized and degraded (Phonekhampheng, 2008). Although the African Catfish has protein requirements like other animals, their requirement for a source of non-specific nitrogen and indispensable amino acids is more critical (Robinson *et al*., 2001). Being euryphagic in nature, catfish feeds predominantly on fish (Viveen and Gophen, 1987), having a propensity toward a carnivorous feeding habit suggesting that *C. gariepinus* has a relatively high dietary protein requirements (Phonekhampheng, 2008; FAO, 2015). According to De Silva and Anderson (1995), because the use of protein as an energy source is expensive, the diet of catfish should be balanced to ensure that the balance of adequate levels of nonspecific nitrogen, amino acids and non-protein energy are supplied in right proportions for cost effectiveness. Studies have been done to determine the protein and amino acids requirement of catfish for several years, yet there is still difficulty in establishing the level for most cost-effective growth (Conceição *et al*., 1998; Robinson *et al*., 2001). The difficulty in preferring specific protein requirements optimum for all situations results because of the several factors which affect dietary protein requirements. These factors include: water temperature; feed allowance; size of fish; amount of non-protein energy in the diet; protein quality; available natural foodstuffs; and management practices.

Although data from several studies indicates that the dietary protein requirement of catfish ranges from 25-50% (Robinson *et al*., 2001), Agricultural Development and Coordination Programme (1983) stated that the best growth rates and food conversions are said to be achieved

with diets containing 35-42% crude protein and a calculated digestible energy level of 12 kJ g-1. Even protein levels as low as 16% may be adequate for growout of food-sized catfish when the fish is fed to satiety (Robinson *et al.*, 2001). According to FAO (2015), the dietary protein requirement for the larvae and the early juveniles is 55% while for the fingerling (grow out phase), it is 40-43%. The rationale behind the considerations of optimum dietary protein level is driven by economics as much as rate of gain. Hence, for profit maximization, the optimum dietary protein level should be changed as fish and feed prices change.

The quantity and availability of amino acids differ according to feedstuff and ingredients. Some ingredients have high levels of nitrogen or proteins but cannot be utilized properly by the fish. This according to Ugwuoke and Ugwu (2013) could be as a result of the presence of some anti-nutritional factors or the absence of some essential amino acids. This is usually the case when non-conventional feedstuff not normally included in catfish diet are used (Francis *et al.*, 2001). Animal protein on the other hand has a full complement of the essential amino acids than plant protein. Major protein ingredients or feedstuffs include fish meal, meat scrap, blood meal, maggot (meal), animal offal, soybean meal, cotton seed meal, groundnut meal, sunflower meal, poultry by-products. Pantazis (1999) determined the quantitative requirements for essential amino acids in feed for *Clarias gariepinus*. His reported values, as a percentage of dietary protein, were: Histidine 1.39, Isoleucine 1.56, Leucine 4.87, Lysine 4.49, Phenylalanine 4.56, Threonine 2.04, Tryptophan 2.59 and Valine 2.08. These requirements were however determined using the daily deposition technique (Jauncey *et al.*, 1993). Dietary nutrient levels for the *Clarias gariepinus* as recommended by Aquaculture Development and Coordination Programme (ADCP, 1983) for different ages are shown in Table 4.

Table 4: Recommended dietary nutrient levels (in DM) for African catfish (*Clarias gariepinus*)

Component	Fry and fingerlings	Growers	Broodstock
Digestible protein, %	35-40	30-35	35-40
Digestible energy (Kj/g)	12.5-16.7	10.5-14.6	12.5-16.7
Ca (min-max),%	0.8-1.5	0.5-1.8	0.8-1.5
P (min-max),%	0.6-1.0	0.5-1.0	0.6-1.0
Methionine + Cystine (min), %	1.2	0.9	1.0

Lysine (min),%	2.0	1.6	1.8

Source: ADCP, 1983.

A more recent recommendations by several authors of the nutritional requirements of *Clarias gariepinus* have been made and are presented in Table 5.

Table 5: nutrient requirement of *C. gariepinus* fish as reported by several researchers

Nutrient	Quantity	References
Crude protein (% min)	40ό43	1, 2, 3, 4, 5, 6, 7, 8, 9
Least cost/appetite feeding protein requirement	35-38	1, 6
Crude lipid (% min)	10-12	1, 2, 6, 7
Carbohydrate (% recommended)	15-35	1, 6, 7, 10, 11, 12
Digestible energy (min, kJ/g)	14-16	1, 2, 13
Metabolizable energy (min, kJ/g)	13	2
Gross energy (min, kJ/g)	22-24	2, 4, 7, 10
Protein to energy ratio (mg/kJ)	22-30	1, 2, 4, 7, 10
Lipid to carbohydrate ratio (g/g)	2.47	6

Source: 1: Uys (1989); 2: Machiels and Henken (1985); 3: Machiels and Henken (1987); 4: Degani, Ben-Zvi and Levanon (1989); 5: Fagbenro, Nwanna and Adebayo (1999); 6: Ali (2001); 7: Pantazis (1999); 8: Ali and Jauncey (2005b); 9: Ali and Jauncey (2005c); 10: Ali and Jauncey (2005a); 11: Balogun and Ologhobo (1989); 12: Heinsbroek, Van Thoor and Elizondo (1990); 13: Yilmaz *et al*. (2006).

The dietary protein-energy ratio recommended for *Clarias gariepinus* catfish have also been reported and these are shown in Table 6.

Table 6: Dietary protein and energy requirements for African catfish (*Clarias gariepinus*)

Protein (%)	Energy (kJ/g)	P/E (mg/kJ	Reference
>40	13 ME	31	Machiels and Henken (1985)
40-42	14-16 DE	26-29	Uys (1989)
40	11-13 GE	31-36	Degani *et al*. (1989)

GE = gross energy; ME= metabolizable energy; DE= digestible energy P/E= protein-energy ratio

2.3.5 Vitamins and Minerals

Vitamins are a heterogeneous group of organic compounds required by an animal in small quantities for growth and maintenance of animal life (Robinson *et al*., 2003). Although the body produces some vitamins, the majority of the vitamins are either not synthesis by the body or are not synthesized at the rate sufficient to meet the animaløs need. They differ from the major classes of food nutrients in that they greatly vary in chemical structure are present in trace amounts in feed ingredients and are required by fish in minute quantities (Oluwatosin, 2011). According to Oluwatosin (2011), 15 vitamins have been biologically isolated with their essentiality differing according to animal species, the growth rate of the animal; feed composition; and the bacterial synthesizing capacity of the gastro-intestinal tract. The amounts and quality of vitamins that catfish need have been fairly well determined in both laboratory and pond studies (National Research Council, 1993). Commercial catfish feeds are generally supplemented with a vitamin premix that contains enough of all essential vitamins to meet the requirement and make up for losses from feed processing and long duration storage (Oluwatosin, 2011). Vitamin loss during storage is not a major factor if the feed is stored for a short time (Robinson, 1989). Vitamins present in feedstuffs have usually not been considered during feed formulation because their bioavailability is not known (Robinson *et al*., 2001). Apart from the vitamin premix used during feed formulation, natural food organisms may also be a source of vitamins for catfish. Evidence of this was shown with respect to zooplankton collected from commercial catfish ponds that contain all vitamins, some in relatively high amounts (Robinson *et al*., 2001). Studies carried out by these authors have shown the amount of the vitamin-premix to use and even certain vitamins that can be removed from the vitamin-premix without affecting the performance of the fish. Nevertheless, some vitamins like vitamin C are given in high amounts (about 2,000ppm) during late winter or early spring, possibly to enhance the immune system because of their antioxidant properties (Robinson *et al*., 2001).

Minerals are required by catfish for metabolism and skeletal structure. They are also required for osmotic balance between their body fluids and their environment (Robinson *et al*., 2001). Although mineral studies with fish are difficult to perform, mineral needs of catfish have been suggested, and the signs of mineral deficiency have been described (Robinson and Li, 1996). Although fourteen minerals are considered essential to catfish production, Phosphorus is particularly important in fish feeds because fish require a large amount of it for growth, bone mineralization, lipid and carbohydrate metabolism (Oluwatosin, 2011). Feedstuffs, especially those from plants, are poor sources of phosphorus, and fish do not get enough phosphorus from pond water (as is the case with calcium). Studies show that phosphorus are mainly present in

plant sources in the form of phytate which is poorly utilized by fish. However, their utilization could be efficiently enhanced by adding phytase enzymes to such diets (Robinson *et al*., 2001). Also, pollution of the water with excess Phosphorus could be critical, resulting to eutrophication (Oluwatosin, 2011).As a result, African catfish feeds are usually supplemented with phosphorus in the form of Dicalcium and defluorinated phosphates. In addition, sodium, and mineral requirements of fish is normally supplied using common salt. African catfish feed are typically supplemented with a trace mineral premix with enough of all essential trace minerals to meet or exceed dietary requirements of catfish. A trace mineral premix may not be needed in catfish feeds that contain 4 % or more animal protein (Robinson, 1989; Yan *et al*., 2007).

2.4 COCOA (THEOBROMA CACAO)

2.4.1 Origin and Taxonomy

The cocoa (cacao) is a small (468 m tall) evergreen tree in the family, Malvaceae (encyclopedia of life, 2012) which has a significantly high content of theobromine (a substance similar to caffeine). The various parts of the plant have been utilized, namely cocoa beans prepared as chocolate, cocoa bark, cocoa butter, cocoa flower, cocoa pulp and cocoa leaf. The plant is native to the deep tropical regions (forests of the Amazon and Orinoco areas) of Central and South America (https://en.wikipedia.org). According to European Food Safety Authority (2008), cocoa was originally cultivated by Indians living in Mexico and Central America who gave it the name kakaw (Mayan language), and was later called theobroma by the Swedish naturalist, Linnaeus. The name was coined from the Greek words, Theo (God) and broma (food), thus õfood for the godsö as it was believed (Maya mythology) and the Mexican Aztec that cocoa was discovered by the gods in the mountains (https://en.wikipedia.org/wiki/Theobroma_cacao; Callebaut, 2008). It was first introduced by Christopher Columbus to Spain from the Aztecs in 1580 in his fourth voyage as a souvenir and by 1653 cocoa was used in Europe as a medicine rather than a delicious foodstuff (Jalil and Ismail, 2008). The taxonomical classification of the plant is given in Table 7.

Table 7: Scientific Classification of Cocoa

Scientific classification	
Kingdom	Plantae
Unranked	Angiosperms
Unranked	Eudicots

Unranked	Rosids
Order	Malvales
Family	Malvaceae
Genus	Theobroma
Species	**T. cacao**

Source: www.wikipedia.com

2.4.2 Production and distribution

Theobroma cacao is characterized by three main cultivar groups: Criollo, Forastero and Trinitario, which have been widespread in the sub-humid tropics. The tree takes about 7 years to mature and produces for 20 years, with an annual yield of around 50 to 60 pods (Pohlan and Perez, 2012). Cocoa is one of the worldøs most valuable crops, produced in countries in the belts between 10ºN and 10ºS of the Equator (often called the õcocoa beltö) requiring a warm and humid climate (International Cocoa Organization, 2015). It is grown in 58 countries, cultivated on 8.2million hectares, and worth over US$4 billion annually (Pohlan and Perez, 2012). Rainfall and temperature are important in encouraging optimum growth of the crop. Cocoa plants respond well to relatively high temperatures, with a maximum annual average of 30 - 32ºC and a minimum average of 18 - 21ºC.

Table 8 shows cocoa beans production statistics from 2011- 2013, by region. In 2013, about 235,000 metric tons of cocoa beans were produced in Nigeria during 2014/ 2015 period.

Table 8: Global cocoa bean production from 2011-2013

S/N	Country	**2011 (**tonnes)	**2012 (**tonnes)	**2013 (**tonnes)
1	Cote døIvoire	1,511,255	1,485,882	1,448,992
2	Ghana	712,200	879,348	835,466
3	Indonesia	700,020	740,500	777,500
4	Nigeria	391,000	383,000	367,000
5	Cameroon	240,000	268,941	275,000
6	Brazil	248,524	253,211	256,186

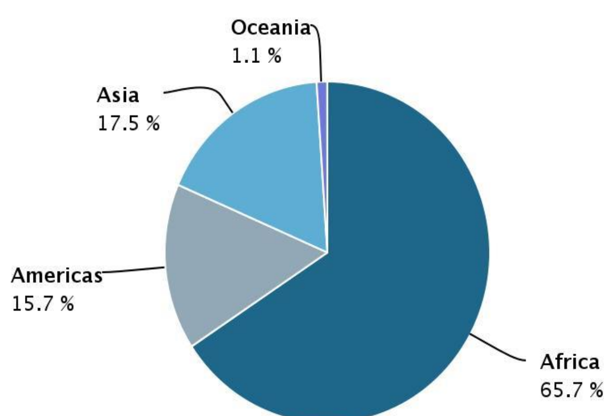
Source: www.faostat3.fao.org

The major producers of cacao beans are generally African, South East Asian and Central and South American countries as shown in figure 1. According to FAO (2015) the world output of cocoa bean is placed at 4,585,552 tonnes with Africa having about 3,014,874 tonnes in 2013.

Africa dominates the world production with 65.7% followed by America with 15.7%. Ivory Coast dominates among global producers with a large share of the world total output (1,449 metric tonnes) in 2013 representing over 48% of the total produce from Africa. The Ivory Coast, Ghana, Indonesia and Nigeria represent the world largest cocoa producing countries in that descending order.

The world distribution of cocoa production is also shown in figure 1 to elucidate the proportion of the world cocoa output generated from each region.

Figure 1: World production distribution of cocoa production in 2013



Source: www.faostat3.fao.org

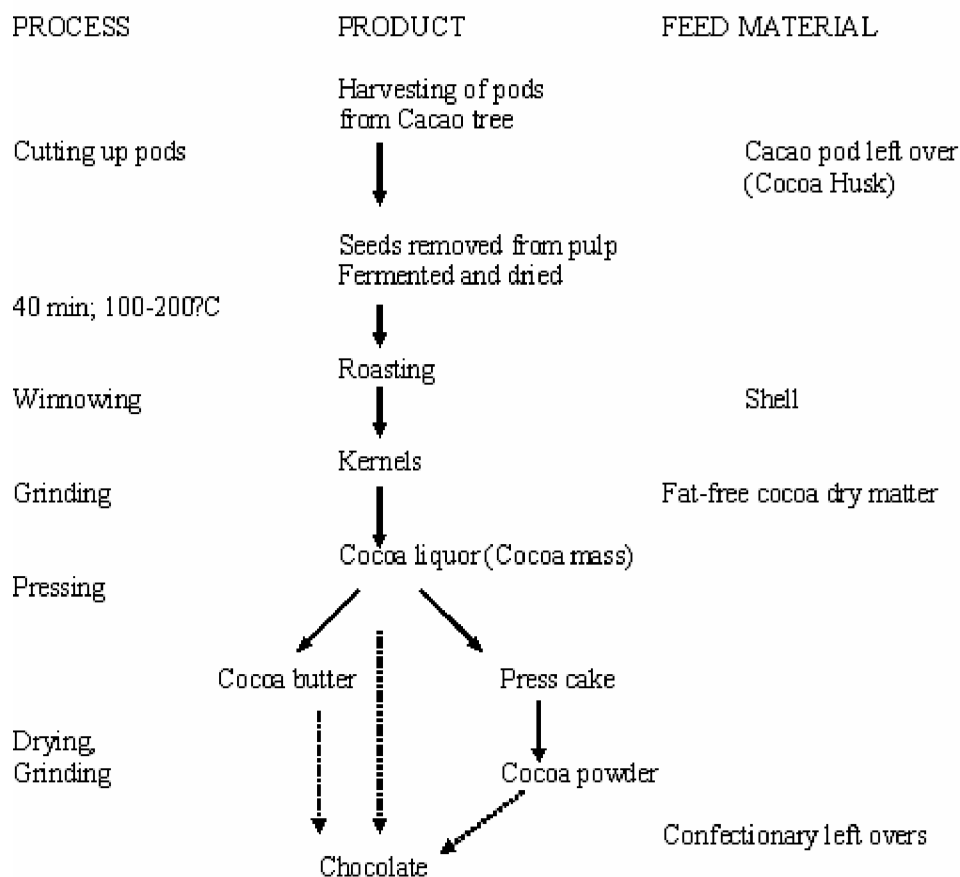
2.4.3 Products from cocoa

There are different products that are derived from cocoa or for which cocoa cultivation and production is done. Cocoa beans are mostly processed into chocolate cocoa products and wide range of intermediate products such as cocoa liquor, cocoa butter, cocoa cake and raw cocoa powder (Afoakwa *et al*., 2011). The major product for which cocoa is cultivated is the beans, which are contained in large red or yellow pods growing directly on the stems and branches of the tree (www.feedipedia.org), whereas cocoa husk (the remains of the cacao pod when beans have been harvested) is the by-product of cocoa production, cocoa meal, cocoa shells, and discarded confectionary are the major by-products of cocoa manufacturing (EFSA, 2008). When the pods are ripe they are cut from the tree by hand, cut open and the fresh seeds (beans) with adhering pulp (mucilage) are taken out of the pod and kept to ferment in a heap or a box. The fermentation takes 4 to 6 days and it is followed by extraction and drying of the beans which is usually done in the sun for about one week (Feldman, 1998). The dried beans are then the starting material for cocoa products production. The left-over pods and pulp is a potential source

of livestock feed or soil fertilization material and are usually available at the site were cacao trees are grown or processed. A simplified diagram illustrating the processing methods and products associated with the manufacture of chocolate is given in Figure 2. Cocoa processing follows series of activities including fermentation, roasting and grinding. Firstly, they are roasted to develop flavour and aroma. On cooling, the beans are cracked and the shell winnowed away, leaving the cracked seed kernels known as the nib. The nib is ground to give cocoa mass or chocolate liquor, from which the cocoa fat (cocoa butter) is extracted by pressing. The resulting cake is pulverized into cocoa powder. The pods, being rich in potassium, are left in the field to rot and fertilize the cocoa trees (http://www.feedipedia.org).

Cocoa powder is essentially used in flavouring biscuits, ice cream and other dairy products, drinks and cakes and in the manufacture of coatings for confections and frozen desserts (Afoakwa *et al*. 2007; Pandey and Singh 2011; Frost *et al*. 2011; Rossini *et al*. 2011) and in the beverage industry, for the preparation of chocolate milk (Afoakwa *et al*., 2011). Cocoa butter is used in the manufacture of chocolate confectionery, soap and cosmetics (Ntiamoah and Afrane, 2008; Schumacher *et al*. 2010). Other by-products such as cocoa pulp juice is also fermented to produce industrial alcohol and alcoholic beverages such as brandy and wine (Jayathilakan *et al*. 2011). The pod husks and shells are used for the preparation of animal feed and fertilizer (Ntiamoah and Afrane 2008). Also, when excess cocoa is produced, surpluses may be sold for livestock feeding under the name of cocoa meal.

Figure 2: products associated with the manufacture of chocolate



Source: European Food Safety Authority (EFSA, 2008)

2.4.4 Chemical composition of the cacao fruit, cacao bean and cacao products

Cocoa and cocoa products contain high amounts of the polyphenol flavonoid monomers (catechin and epicatechin) up to tetradecamers (Kelm *et al.*, 2006; Tomas-Barberan *et al.*, 2007) and other several chemical constituents that could be classified as toxic or antinutritious but may have some positive effects in some cases (Maleyki and Ismail, 2008). These other chemical constituents include methylxanthines (theobromine, caffeine, and traces of theophylline) (Greer *et al.*, 2001; Rios *et al.*, 2003), the biogenic amines (-phenethylamine and tyramine, oxalate, cyanogenic compounds, furfural, anandamides, tannin and trypsin inhibitor (Dodo *et al.,* 1992; Aremu *et al.*, 1995; Rätsch, 1998). The health benefits of cocoa and its by-products are due to their flavonoid contents and several studies have demonstrated their roles. The presence and levels of these compounds (with the exception of the flavonoids and methylxanthines) in the pod is poorly investigated (EFSA, 2008). Also, the characteristic bitter taste of cocoa and chocolate is attributed to the presence of Theobromine, and to some extent, the caffeine content. Cocoa is

also rich in micronutrients such as copper (USDA, 2008). This cocoa copper has been reported to contribute significantly towards human dietary intake (Joo *et al*., 1995). Due to the content of mixtures of bioactive components in cocoa, it may be postulated that there may be direct or indirect synergism between these components in delivering their health properties (Maleyki and Ismail, 2008). Although it was earlier thought that the health benefits of cocoa was as a result of their contents of polyphenols. Studies (Eteng and Ettarh, 2000) have shown that components other than this could also have a role.

Several factors affect the quality and quantity (of constituent compounds) of cocoa and cocoa-based products during production and manufacturing and are of great importance in delivering the best health effects. These factors include the variety of cocoa, the region it is produced (Africa or South America), part or the product, stage of maturity, fermentation, heat treatment, storage, other dietary components (like protein), and physiological factors like bioavailability and antioxidant properties (Wollgast and Anklam, 2000; Misnawi *et al*., 2004 a, b; EFSA, 2008; Maleyki and Ismail, 2008; Rusconi and Conti, 2010; Saltini *et al*., 2013). These factors could greatly reduce the polyphenol content of the selected products

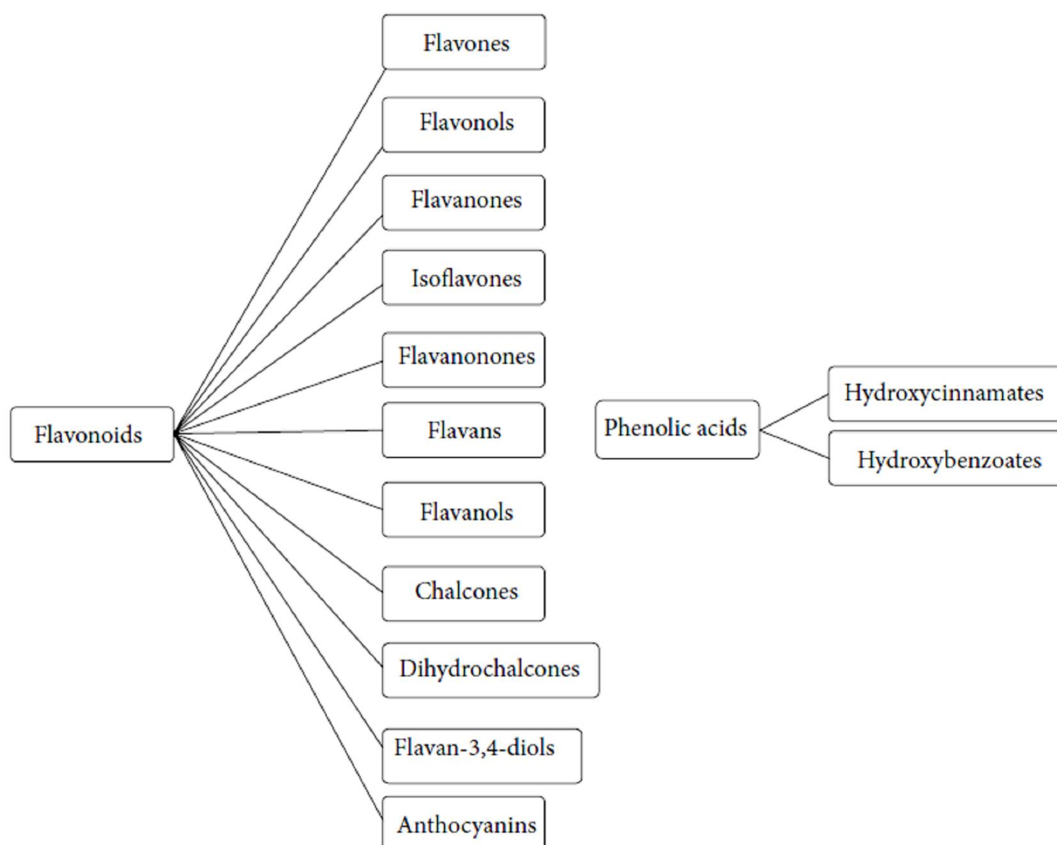
2.4.5 Polyphenols

Cocoa had long been identified as a polyphenols-rich food. The main polyphenol in cocoa or cacao was first identified by Ultée and van Dorsen in 1909. Polyphenols are large and heterogeneous group of biologically active secondary metabolites in plants, where they act as cell wall support materials, colourful attractants for birds and insects, and defence mechanisms under different environmental stress conditions (wounding, infection, excessive light, or UV irradiation) (Hakkinen, 2000). According to Ackar *et al*. (2013), they have been classified into four groups according to their chemical structure: phenolic acids, lignans (recognized as phytoestrogens; flaxseed and flaxseed oil are the main source), flavonoids (the most abundant polyphenols in human diets), and stilbenes (resveratrol is under investigation for its anticarcinogenic properties). The flavonoid group is further divided into: anthocyanins, flavonols, flavanols (catechins in tea, red wine, and chocolate), flavanones (citrus fruit are the main source), flavones, and isoflavones (main source is soya) (Tomas-Barberan, 2012).

However, the three main groups of polyphenols found in unfermented cocoa bean are flavan-3-ols or catechins, anthocyanins, and proanthocyanidins, with average content of 120ó180 g/kg (Misnawi *et al*., 2004 a; b). The main polyphenol compound in fresh cocoa bean is ( )-epicatechin, with average content of 21ó43 mg/g of defatted sample, followed by (+)-catechin as

well as dimers and trimers of these compounds (Jalil and Ismail, 2008). Apart from flavonols (epicatechin and catechin) and tannin, other polyphenols include flavonoids and leucoanthocyanins which are present as glycosides, procyanidin B1, B2 and C1 (Maleyki and Ismail, 2008). Complex alteration products of catechin and tannin give brown and purple colour to cocoa bean (Jalil and Ismail, 2008). As stated earlier, several factors especially processing, affect the content and availability of polyphenols in cocoa products. These alterations in the chemical constituents during the processing activities are necessary for maintaining satisfactory aroma of cocoa. However, processing are nowadays conducted in such a manner as to preserve as much polyphenols as possible while maintaining satisfactory aroma. Cocoa powder have been shown to contain high levels of polyphenols and antioxidant properties but these are destroyed by alkalization (a processing method) (Crozier *et al*., 2012). The basic classification of flavonoids are given in figure 3.

Figure 3: basic classification of flavonoids and phenolic acids



Source: Jakobek et al. (2007)

2.4.5.1 Bioavailability of polyphenols

The bioavailability of polyphenols in the body is affected by the chemical structure of polyphenols, food matrix, factors related to food processing, and interactions with other constituents in diet, as well as with some host related factors (genetic aspects of individuals, gender and age, disorders, physiological condition, microbiota metabolism and enzyme activity in the colon) (DøArchivio *et al*., 2010; Tomas-Barberan, 2012). Although polyphenols are found in different food sources (fruits and vegetables), some polyphenols are specific to particular food and some are found in all plant products, so that, generally, food is considered to contain complex mixtures of polyphenols (DøArchivio *et al*., 2010).

Isoflavones and phenolic acids have the highest absorption in the intestine, followed by catechins, flavanones, and quercetin glucosides, whereas proanthocyanidins, anthocyanidins, and galloylated tea catechins are poorly absorbed (Han *et al*., 2007). After absorption, polyphenols are conjugated to glucuronide, sulphate, and methyl groups in the gut mucosa and inner tissues, where epicatechin and epigallocatechin are mostly present in the glucuronide and sulfate conjugates (Ackar *et al*., 2013). Absorption of epicatechin and catechin in the intestine averages between 22% and 55%, while dimers and trimers are poorly absorbed (less than 0.5%).

Procyanidins cross intestinal barrier and are transported to liver, where they undergo methylation, glucuronidation, and sulfation which result in antioxidant capacity (Han *et al*., 2007). Polyphenols that reach colon are fermented into phenolic acids of low molecular weight by microflora population (Han *et al*., 2007).

In humans, epicatechin from chocolate is rapidly absorbed with its plasma levels detected after 30 min of oral digestion, peaking after 2-3 h and returning to baseline after 6ó8 h. The amount of the polyphenol influences its absorption. Generally, it can be said that the smaller the polyphenol, the higher the concentration in blood and the higher the chance it will reach its target organ in the body (Cooper *et al*., 2008).

Chirality (referring to enantiomers or optical isomers) might also influence bioavailability of ÷polyphenols (+)- form of catechin is almost 10 times more absorbed than ( )- form (Cooper *et al*., 2008). Sugars and oils are known to increase bioavailability of polyphenols, while proteins, on the other hand, decrease it (Tomas-Barberan, 2012). In his research, Neilson *et al*. (2010) showed that milk proteins and sucrose modulate metabolism, plasma pharmacokinetics, and bioavailability of catechins from chocolate confections. They found that milk proteins reduce bioavailability of epicatechin in chocolate confectionary. In addition, Serafini *et al*. (2003) reported inhibition of in-vivo antioxidant activity of chocolate by milk addition either during manufacturing process or during ingestion. However, this effect was not observed in chocolate beverages (Nelson *et al*., 2010).

2.4.5.2 COCOA POLYPHENOLS AND HEALTH

Antioxidant Properties

Many researches have shown that polyphenols and polyphenol-rich foods play an important role in health preservation due to their antioxidant properties (Han *et al*., 2007; Cooper *et al*., 2008; Awe *et al*., 2013). Although polyphenol content can be positively correlated with antioxidant properties as measured by oxygen radical absorbance capacity (ORAC) (Adamson *et al*., 1999), all polyphenols are not likely to possess antioxidant properties in-vivo as is the case in-vitro (Maleyki and Ismail, 2008). It was reported from an *in-vivo* study that epicatechin from cocoa could enhance the antioxidative activity of blood plasma (Baba *et al*., 2000) and decreases 8-isoprostane in a dose dependent manner (Rein *et al*., 2000; Wang *et al*., 2000). The presence of epicatechin (12-fold from baseline) leads to significant increase in total antioxidant capacity of the plasma and decreases plasma thiobarbituric acid reactive substances.

The antioxidant activity of cocoa and chocolate was shown to be correlated with their catechin and procyanidin contents (Wan *et al*., 2001). Polyphenols can act as proton donor-scavenging radicals (Rice-Evans *et al*., 1997) and inhibitors of enzymes that increase oxidative stress, chelate metals, bind carbohydrates, and proteins (Helm *et al*., 2002). Verstraeten *et al.* (2004) reported that the chemical structures of flavonols and procyanidins were important for their antioxidant activity as they possess both free radical trapping and chelation of redox-active metals properties (Verstraeten *et al*., 2004). Also, flavonoids and procyanidins were found to prevent lipid oxidation through interaction between lipid forming membranes and the adsorption to the polar lipid headgroups (Verstraeten *et al*., 2005). These properties enable them to act as anticarcinogenic, antiinflammatory, antihepatotoxic, antibacterial, antiviral, and antiallergenic compounds (Arts and Hollman, 2005; Vita, 2005). According to Maleyki and Ismail (2008), several reports have failed to show these promising health benefits of cocoa.

Cardiovascular Anti-Carcinogenic Effects

Studies (Murphy *et al*., 2003; Heiss *et al*., 2005; Cooper *et al*., 2008; Hollenberg *et al*., 2009 and Djousse *et al*., 2011a; b) established a relationship between high consumption of cocoa beverages and dark chocolates and very low blood pressure levels, reduced frequency of myocardial infarction, stroke, diabetes mellitus, lower prevalence of cardiovascular diseases, improved platelet aggregation and cancer. (Murphy *et al*., 2003) reported that supplementation of cocoa for four weeks significantly improved the platelet function among healthy subjects. Most observations of the effect of cocoa polyphenols on cardiovascular health have been made on their effect on antioxidant status, endothelial function, inflammatory production, nitric oxide bioactivity and platelet function (Rimbach et al., 2009) as these factors are associated with coronary diseases (Engler and Engler, 2004). Research of Heiss *et al.* (2003) showed that a single dose of cocoa drink enhanced flow mediated dilation (FMD), increased nitric oxide in human plasma and improved endothelial dysfunction. Procyanidins extracted from cocoa caused endothelium-dependent relaxation (EDR) through activation of nitric oxide synthase activity in rabbit aortic rings *in vitro* (Karim *et al*., 2000). *In vivo* studies have shown that dark chocolate, cocoa powder and cocoa liquor suppressed the development of atherosclerotic lesions and inhibited atherosclerosis (Kurosawa *et al*., 2005; Vinson *et al*., 2006). High-flavonoid content dark chocolate (containing 259 mg polyphenols) significantly improved FMD in healthy subjects compared to low-flavonoid chocolate (containing trace amount of polyphenols) (Engler *et al*., 2004).

In an in vivo study, Amin *et al*. (2004) indicated that cocoa liquor extract decreased the activity of tumor marker enzymes during hepatocarcinogenesis. Similarly, cocoa powder supplementation significantly reduces the incidence of prostate carcinogenesis compared to positive control (Bisson *et al*., 2008); increased the life span of tumor bearing rats (Maleyki and Ismail, 2008); and reduced prostate hyperplasia by lowering dihydrotestosterone level and prostate size ratio (Bisson *et al*., 2007).

Lipid Profile

Cocoa polyphenols may also be implicated in cholesterol control. Hot cocoa beverage was proven to successfully reduce LDL cholesterol, increase HDL cholesterol, and suppress LDL oxidation in research of Baba *et al*. (2007). Atherosclerotic cholesterol profile (cholesterol: HDL ratio) in patients with diabetes was improved after 8-week chocolate consumption without affecting weight, inflammatory markers, insulin control, or glycaemic control (Mellor *et al*., 2010). Waterhouse *et al*. (1996) reported that polyphenols from chocolate inhibited LDL oxidation by 75%, compared to 37ó65% of red wine (Cooper *et al*., 2008). In addition, Vinson *et al*. (1999) reported that dark chocolate had higher quality of phenol antioxidants expressed as IC_{50} for LDL + VLDL oxidation compared to red wine and black tea, with high lipoprotein bound antioxidant activity, which is very important in prevention of heart diseases. According to Mursu *et al*. (2004) HDL cholesterol increased after 3-week consumption of dark and polyphenol-rich dark chocolate. Total and LDL cholesterol were decreased after 15- day consumption of polyphenol-rich dark chocolate by 6.5% and 7.5%, respectively (Grassi *et al*., 2005; Grassi *et al*., 2008). It was reported that regular consumption of dark chocolate (up to 7 days) decreased LDL cholesterol and increased HDL (Hamed *et al*., 2008). On the other hand, Kurlandsky and Stote (2006) reported no significant difference in HDL and LDL cholesterol levels between õchocolate consumingö and control group. However, large age difference between control and õchocolate consumingö group may have influenced these results. Almoosawi *et al*. (2010) also observed no significant change in total cholesterol level after consumption of dark chocolate.

Blood Pressure and Blood Glucose

Glucose blood levels could be reduced by consumption of dark chocolate; however, the dose and duration of treatment seem to significantly influence the effectiveness of treatment. As opposed to short-term treatments of Stote *et al*. (2007; 2012) and low-dose treatment of Taubert *et al*. (2007), long-term high-dose treatment of Almoosawi *et al*. (2010) proved to be effective. Researches showed that short-term administration of dark chocolate increases insulin sensitivity

both in healthy (Grassi *et al.*, 2005) and in glucose-intolerant hypertensive people (Grassi *et al.*, 2008).

Insulin response and blood pressure could be linked with the regulation of nitric oxide production by dark chocolate flavanols. Increased generation of nitric oxide (NO) and reactive oxygen species (ROS) in the vessel wall in response to dietary isoflavones enhances the activity of antioxidant defense enzymes in endothelial and smooth muscle cells (probably owing to estrogenic properties of isoflavones) by activation of signaling pathways that increase NO bioavailability and regulate phase II and antioxidant enzyme expression via the redox sensitive transcription factor Nrf2 (Sow and Mann, 2010; Ried *et al.*, 2010). Investigations carried out by Ried and coworkers showed that flavonol-rich chocolate may have a small but statistically significant effect on lowering blood pressure by 2-3 mm Hg in the short term (Ried *et al.*, 2010; Ried *et al.*, 2012).

Cognitive Enhancement

Flavanol-rich cocoa increases blood flow to key areas of brain increasing blood oxygenation level-dependent response to cognitive task switching paradigm in healthy young people (Francis *et al.*, 2006) and could be useful in treatment of cerebrovascular flow (CBF) dementia (Fisher *et al.*, 2006), Alzheimerøs disease (Patel *et al.*, 2008), and stroke (Soron *et al.*, 2008). Daily cocoa extract administration protect the brain from cognitive impairments by preventing the overproduction of free radicals after heat exposure as observed in rats (Rozan *et al.*, 2006; Bisson *et al.*, 2008).

In addition, Chandranayagam *et al.* (2013) reported that tannin-rich chocolate can be considered as functional food which effectively antagonizes adverse effects of arsenic intoxication. However, this research was conducted on Sprague Dawley rats and should yet be confirmed by research on humans

2.4.5.3 Methylxanthines

In addition to polyphenols, cocoa is also rich in methylxanthines, namely caffeine, theobromine, and theophylline (Greer *et al.*, 2001; Rios *et al.*, 2003). Found in dark chocolates, these compounds are responsible for chocolate cravings (Smit and Blackburn, 2005). The fresh unfermented cacao beans contain 14-38 g theobromine and 1-8 g caffeine per kg seed material on a dry weight basis with theophylline present in trace amount (Sotelo and Alvarez, 1991; Greer *et al.*, 2001; Naik, 2001; Rios *et al.*, 2003;). Hence, the caffeine content is only about 10-15% of the theobromine content. Genotype highly affects the amount of the methylxanthines in cocoa.

Hence, African cacaos contain less caffeine and more theobromine than cacaos from South America (Matissek, 1997).

Although most studies have attributed the beneficial effect of cocoa or cocoa products on the health to polyphenols (Cooper *et al*., 2008), it should be noted that cocoa and cocoa products in addition to their high content of polyphenols, are also rich in methylxanthines compounds. To this effect, the question of whether the presence of methylxanthines enhances or reduces the health benefits of the cocoa flavonoids is yet unanswered, as there has been conflicting evidence in favor or against the subject (Maleyki and Ismail, 2008). Hence further studies are necessary to establish the possible synergistic interactions between flavonoids and methylxanthines. The effects of theobromine is studied to a much lesser extent than those of caffeine but it is however known that this molecule exerts some positive effects in different human pathologies (Pinilla *et al*., 2015). According to these authors, the combination of caffeine and theobromine in cocoa may have the expected methylxanthine-derived benefits without the side effects reported for caffeine. Interestingly, the main action mechanism of caffeine and theobromine consists of blocking adenosine receptors and inhibiting phosphodiesterases (Pinilla *et al*., 2015). Contrary to general perceptions, caffeine and theobromine are not addictive substances (National Institute on Drug Abuse, 2014) and are not also enlisted as doping substances by the World Anti-Doping Agency (World Anti-Doping Agency (WADA), 2014).

2.4.6 THEOBROMINE AND CAFFEINE

Theobromine is a colourless and odourless substance (melting point 35.7^{o}C) with a slightly bitter taste that is naturally present in all parts of the seed and in small quantities in the pod, most likely as a component of the chemical defence mechanism of the cocoa plant (IARC 1991; Windholz, 1983; Aneja and Gianfagna, 2001). It is a psychoactive compound without diuretic effects (Maleyki and Ismail, 2008). Sotelo and Alvarez (1991) reported that theobromine content of Theobroma spp. varies with the parts of the fruit, being high in the seed (shown above), about 1 g/kg in the hull and around 0.2 g/kg in the shell. Theobromine and caffeine content was also reported to vary with the cycle of growth and development of the cocoa pods (Senanayake and Wijesekera, 1971). During fermentation, the methylxanthine concentrations decrease by around 25-40% due to exudation from the nibs to the shells and to the surrounding seatings (Ziegleder and Biehl, 1988).

Methylxanthines particularly caffeine, could exert pro-oxidant properties, and caffeine, theobromine, and theophylline exerted antioxidant activity and protective ability under

physiological conditions (Lee, 2000). However, Vinson *et al.* (1999) have reported that theobromine and caffeine were neither pro-oxidant nor antioxidant. When taken in modest quantities, theobromine acts as a stimulant like caffeine but intake of more than 0.0279 kg per body weight is injurious to animals (Menon, 1982)

It has been shown that supplementation of 3% and 15% cocoa powder (containing 56 to 265mg of theobromine) significantly reduced body weight and lipid profiles in hyperlipidemic rats (Eteng and Ettarh, 2000; Eteng *et al*., 2006). It is said that theobromine reduces lipid profile by activating the hormone sensitive lipase which hydrolyses triacylglycerols from adipose tissues to fatty acids and glycerols in the plasma (Granner, 1990). Theobromine and its synthetic analogues have been shown to have a positive effect on cancer and hence used for cancer therapy (Chang *et al*., 1993; Gil *et al*., 1993; Clark *et al*., 1996; Barcz *et al*., 1998).

On the other hand, theobromine has been reported to negatively affect reproductive health and up to 250mg/kg body weight, can result to vacuolation within the sertoli cells, alter spermatid shape and inhibit the release of male spermatids in male rats (Wang *et al*., 1992). Similarly, high dose of cocoa extract containing theobromine was reported to greatly alter testicular structure (Maleyki and Ismail, 2008), cause testicular atrophy and aspermatogenesis in rats (Weinberger *et al*., 1978). However according to this author, the atrophied testes had hyperplastic leydig cells which was accompanied by increased testosterone secretion. In addition exposure of theobromine to male rats resulted in decreases in cauda epididymal sperm reserve, seminiferous tubule fluid volume, lactate concentration in seminiferous tubule fluid, weight of prostate and seminal vesicle, binding activity of androgen binding protein, and reduced content of the androgen binding protein in seminiferous tubule fluid (Wang and Waller, 1994; Funabashi *et al*., 2000).On the contrary, feeding male dogs theobromine (from 25 to 150mg/kg b.w. per day) up to a year failed to cause testicular atrophy (Gans *et al*., 1980). Theobromine may be toxic to some animals including pets (eg dogs and horses) but not as toxic in humans (Gans *et al*., 1980; Smit, 2011). This differences could be attributed to the species differences in the rate of theobromine catabolism (Adamafio, 2013). Other detrimental effects of theobromine include a reduction in the thymus weight, a slow heart rate, excitability, digestive complications and impaired growth (Howell *et al*., 1997; Alexander *et al*., 2008). Cocoa contains very low caffeine when compared to coffee and tea (USDA, 2008). However it has been shown to have a similar or even stronger negative effect on the testes than theobromine when fed at up to 300mg/kg b.w. (Gans, 1984).

2.4.6.1 Theobromine toxicity

Having shown the properties and qualities of theobromine as a content of feed, it is important to restate that theobromine is the major substance responsible for the detrimental effects of feeding cocoa and its by-products to animals. This was established by demonstrating the toxic effect of pure theobromine on laboratory animals and livestock (Adamafio, 2013). According to Adamafio (2013), the aberrations caused by theobromine can be divided into broad categories which include reproductive and development toxicity. These have being discussed earlier.

2.4.6.2 Detheobromination methods

To improve the use of cocoa by-products in animal production, attempts have been made to detoxify these products using both physicochemical and biological methods. This have resulted to varying degrees of success.

- Physicochemical treatment: these include methods as boiling, treatment with hot water (90°C) or alkali (cocoa pod ash solution) which have been reported to reduce the nutritional components of the materials (Odunsi *et al*., 1999; Olubamiwa *et al*., 2006).
- Biological treatment: this involves the use of isolated atoxigenic microorganisms that express theobromine degrading enzymes and have the capacity to colonize the cocoa by-products (Adamafio, 2013).
- Fungal, yeast and bacteria treatment: this involves the use of filamentous fungi, *Pleurotus pulmonarium* (Muhammed *et al*., 2000); the yeast, *Candida krusei* (Adamafio *et al*., 2012), and the bacteria, *Pseudomonas putida* (Yu *et al*., 2009) to ferment and reduce theobromine content. These organisms however, have demonstrated varying degrees of success.

2.4.7 Proteins

In addition to polyphenols and methylxanthines, cocoa is also rich in proteins. The crude protein content of cocoa bean is less than 25%. It is low in the amino acid, methionine, which is an essential amino acid needed to make a complete protein (Rucker, 2009). The amino acid and peptide content of cocoa are responsible for the taste and flavour precursor formation (Voigt *et al*., 1994a; b). There are basically four types of proteins contained in Cocoa beans which include: albumins, globulins, prolamin, and glutelin. Of these, albumin constitutes the major protein fraction (Zak and Keeney, 1976a; b). Albumin and globulin fractions accounted for 52% and 43% of total bean proteins, respectively (Voigt *et al*., 1993).

There are few studies on the contribution of cocoa peptides towards health, although bioactive peptides have been reported to possess antihypertensives, antithrombotic, hypocholesterolemic,

hypotriglyceridemic, and antiobesity effects (Buyukpamukcu *et al.*, 2001). The antioxidant activity of cocoa has been attributed to their histidine, tyrosine, methionine and cysteine content (Maleyki and Ismail, 2008). Among these amino acids, histidine has shown strong radical scavenging activity due to the decomposition of its imidazole ring (Yong and Karel, 1978). Cocoa and maize are complementary nutritionally and this is important in diet formulation in animal husbandry. As stated by Rucker (2009), when consumed together, the amino acid deficit in cocoa (methionine) is supplied by their rich presence in corn, while the low levels of lysine and tryptophan in the corn are improved by cocoa.

In addition, hydrophobicity of peptides also appears to be an important factor for their antioxidant activity, due to increased interaction with hydrophobic targets (e.g. fatty acids) (Chen *et al.*, 1998). However, the health effects of peptides in humans and the optimal plasma levels remain to be elucidated (Erdman *et al.*, 2008).

2.4.8 Vitamins and Minerals

As earlier stated, the health properties of cocoa and cocoa products are not solely attributed to their polyphenol contents, but also to other components such as methylxanthines (caffeine and theobromine), peptides, and minerals (Maleyki and Ismail, 2008). Although previous studies seem to underestimate the contribution of minerals and peptides in cocoa and cocoa products towards health benefits, Steinberg *et al.* (2003) showed that minerals are one of the important components in cocoa and cocoa products.

Cocoa and cocoa products have been reported to contain relatively higher levels of magnesium than black tea, red wine, and apples (USDA, 2008). Cocoa could possibly be the highest source of magnesium (known as the relaxer mineral) (http://realrawfood.com/cacao-info). According to Rucker (2009), cocoa is an excellent source of most essential minerals, especially calcium, copper, iron, manganese, magnesium, phosphorus, potassium, chromium, and zinc when expressed as amounts per kilocalorie. The human dietary requirements of copper, iron, and magnesium are exceeded several fold on caloric levels by cocoa (DFI, 2003)

Cocoa is not an important source of vitamin. Its content of vitamin A is negligible; the quantity of vitamin C is very low, and the B-group vitamins are also low and decline further in alkalized cocoa powder as a result of the alkalizing process (deZaan, 2009). The presence of vitamin E (tocopherol), in cocoa butter is an exception. The shell has been known to contain 20-30 IU of Vitamin D_2 (Rucker, 2009). The full nutrition of cocoa powder is shown in Table 9.

Table 9: Nutritional Properties of Cocoa

Nutrients	Amount per 100 grams (Dry powder)
Food energy	225 ó 325 kcal (1000 ó 1500 kJ)
Protein (g)	18 ó 19
Fat (g)	20 ó 25
Carbohydrate (g)	45 ó 55
Fiber (g)	25 ó 35
Ash (minerals) (g)	5 ó 6
Calcium	100 ó 180 mg
Phosphorus	750 ó 1000 mg
Iron	10 ó 15 mg
Magnesium	500 ó 600 mg
Copper	4 ó 6 mg
Zinc	5 ó 10 mg
Potassium	1500 ó 2000 mg
Manganese	3 ó 5 mg
Selenium	15 ó 20 g
Vitamin A Equivalents	10 ó 20 RE (g)
Vitamin E	0.1 ó 0.2 mg
Vitamin K 1	2.5 g
Vitamin B 1 thiamin	0.1 mg
Vitamin B 2 , riboflavin	0.2 ó 0.3 mg
Niacin	2 ó 4 mg
Vitamin B 6 , pyridoxine	0.1 ó 0.2 mg
Folate, total	32 ó 40 mcg
Pantothenate	0.2 ó 0.4 mg
Polyphenols	7 ó 18 g
Theobromine	2 ó 3 g
Caffeine	0.1 g
Cholesterol	0

Source: United State Department of Agriculture (USDA, 1999)

2.5 COCOA BY-PRODUCTS AND THEIR USE IN ANIMAL PRODUCTION

In developing countries scarce protein, energy rich food and feed plants (conventional feedstuffs) are usually shared between man and animals and these results to high cost of animal feed and consequently elevated cost of production (Bamba *et al.*, 2014). Emphasis have been made on the use of non-conventional feedstuffs (agricultural by-products) which are not utilized by man for the formulation of fish diet in order to cut cost (Ashade and Osineye, 2013). There are abundant by-products from cocoa which can be used for feeding animals as only the seeds (bean) are consumed by man (Maleyki and Ismail, 2008). However, by-products of cocoa manufacture

which include cocoa pod husk (estimated at 6.7 million metric tonnes), cocoa bean shell (cocoa hull), cocoa seed meal and cocoa germ have been investigated for their suitability as feed with projected tremendous benefit to animal production (Hamzat and Adeola, 2011; Adamafio, 2013). Unlike the last three which are available in the country where cocoa beans are processed, the cocoa husk is not usually exported from the country where the Cacao tree was grown.

Conclusion from several studies have shown that dietary inclusions of these by-products in levels beyond 10-15% negatively affected growth and reproductive indices (Ettlin *et al*., 1986; Odunsi and Longe, 1995; Yang *et al*., 1997; Adeyina *et al*., 2010). Conditions including vomiting, central nervous system depression, restlessness, diarrhea, muscle tremor, ataxia, haematuria, tachycardia, seizures and even death have been reported in animals when organic mulch composed of cocoa by-products were fed to animals (Tarka Jr. *et al*., 1981; Gans, 1984; Day and Dilworth, 1984; Drolet *et al*., 1984; Alexander *et al*., 2008). However, the susceptibility of animals to the detrimental effects induced by the by-products of cocoa appears to vary accross different aniomal species and ages (Adamafio, 2013). The chemical composition of cocoa husks, shells and meals has been widely reported and are shown in Table 10. The variation in proximate content could be due to year-to-year variation, different varieties being studied, and differences in the preparations of the samples for analysis and the analytical method (Alvarado *et al.,* 1983; EFSA, 2008).

Table 10: The proximate composition (% dry matter) of by-products from the manufacture of cocoa

Proximates	Cocoa husks	Cocoa bean shell	Cocoa bean meal
Crude protein	6.8-10.	14.5-21.6	15.1-28.6
Crude fibre	24-35.4	17.4-20.9	5.8-10.3
Fat	1.6-2.4	3.1-5.2	5.5-16.5
Non-cellulose carbohydrate	46.6	40.6	42.1
Gross energy (MjKg^{-1}DM)	10.7	5.1	7.0

Owusu-Domfeh (1972), Odunsi and Longe (1998), Adamafio *et al*. (2004), Laconi (2009) and Feedipedia (2012)

2.5.2 Cocoa pod husk meal

Cocoa husk represents the left over cocoa pods after the beans and mucilage content of cocoa have been removed. It consist about 2/3 and 3/4 of the total weight of the fruit (average fruit weigh about 400 g) and is usually discarded by local farmers after harvest when the beans have been taken (EFSA, 2008). The search for alternative cheap feedstuffs have seen cocoa-pod

husk/husk meal (CPH/CPHM) as an ingredient which can replace maize and other feedstuffs high in energy owing to its content of pectin and free sugars (Adamafio, 2013). According to Fagbenro (1996), CPHM has a high gross energy (4,293 kcal/kg) content but the digestible (2,146 kcal/kg) and metabolisable (1,690kcal/kg) energy are low. Digestibility decreases as the dietary levels of CPHM increases (Fagbenro, 1992) possibly due to the increasing levels of theobromine, tannins, and phenols (Devendra, 1985).

Several studies have documented the use of dried cocoa pod husk (CPH) in poultry and livestock feeding (Barnes *et al*., 1985; Adomako *et al*., 1985; Donkor *et al*., 1991; Adomako and Osei-Amaning, 1996; Adomako *et al*., 1999; Nworgu *et al*., 2003; Agyente- Badu and Oddoye, 2005; Uwagbboe *et al*., 2010). Increase in egg weight and percentage egg production was reported when CPM was fed to laying hens (Abiola and Tewe, 1991). Similarly, it was shown that replacement of maize (10%) with CPHM (6.5% CPHM) significantly improved weight gain of broiler chickens (Teguia *et al*., 2004). In this study, CPHM was used to replace up to 20% of maize in the diet (13% CPHM in the diet) without any adverse effect on growth. Contrary to this report, feeding broiler chicken up to 10% (Teguia *et al*., 2004) and up to 15% (Odunsi *et al*., 1999) resulted to a reduction in growth performance. Rhule *et al*. (2005) reported that 20% cocoa pod husk diets did not adversely affect the reproductive performance of breeding sows. However according to this authors, 20% but not 10% reduced pigletsø birth weight. Studies on the use of CPHM as an alternative energy source in low cost diet of African catfish (Fagbenro and Sydenham, 1988; Fagbenro, 1995) and Nile tilapia (Pouomogne *et al.,* 1997) have reported up to 45% replacement for maize without any adverse effect on growth and performance. After this level, performance begins to decline due to the increase in fiber level and theobromine (Fagbenro, 1992). A more recent study using Nile Tilapia reported that up to 40% inclusion of CPHM, growth performance was greatly improved, although this resulted to high feacal ouput which was attributed to high crude fiber content and low digestibility (Ashade and Osineye, 2013). However, Falaye and Juancey (1999) reported that diets containing 10 ó 20% inclusion of CPHM caused significant reduction in gross feed conversion efficiency (indicated by decreased feed consumption and weight gain) and apparent digestibility of protein and dry matter in Nile Tilapia. In addition, feeding Cocoa pod endocarp (extracted from cocoa pod) up to 20 % to juveniles of *Clarias gariepinus* did not seem to negatively affect their performance (Adebowale and Olubamiwa, 2008). But above this level (up to 25%) there was an appreciable reduction in growth.

A major setback in the use of this by-product as feed ingredient is the theobromine content which is seen when it is fed at high dietary levels (Hertrampf and Piedad-Pascual. 2012). Anti-nutritional substances such as tannin and lignin in the pod husk also limits its utilization by animals (Olubamiwa *et al*., 2002; Teguia and Beynen, 2005; Ocak, 2005). However, treatments and fermentation with edible mushrooms and chemicals (such as laccase) before use has been reported to result in the reduction of this substances and improve the nutritional value of cocoa pod-husk based animal feeds (Alemawor *et al*., 2009; Mensah *et al*., 2012).

Typical values for the composition of cocoa husks are given in Table 9.

2.5.3 Cocoa bean shell (CBS)

Cocoa bean shell is the thin husk immediately covering the cocoa bean. It is a dry, crisp, slightly fibrous brown husk with a pleasant odour resembling that of chocolate and has been equated to medium quality grass hay in feeding quality (EFSA, 2008). Cocoa bean shell is used as fuel, mulching material, or manure in countries where they are largely produced (Aregheore, 2002; EFSA, 2008). It is a potential dietary ingredient, owing to its availability, non-consumption by humans and nutritional value (NRC 2011). It has been reported that CBS contains 13.2% - 17.7% crude protein and 13.0% -16.1% of fibre (Chung *et al* 2003). The mineral and amino acid profile showed that CBS compares favourably with palm kernel cake (and other by-products of kola, cashew, and tea) and thus can be used as a medium protein source to substitute grain proteins in animal diets (Hutagalung and Chang, 1978; Hamzat and Adeola, 2011). It is reported to have an intermediate buffer value between the protein and cereal feedstuffs and could be utilized both as a medium protein (comparing favourably with palm kernel cake) and carbohydrate sources (Bamba *et al*., 2014). However, the use of CBS in animal feed has been limited by its theobromine content which is as high as 1.3% (the level of which is determined by the preparation method of the cocoa bean) (Gohl, 1981; Olubamiwa *et al*., 2001; Olubamiwa *et al*., 2002; Olubamiwa and Hamzat, 2005). Originally, the shell contains low theobromine. This is however acquired from the nib during fermentation (EFSA, 2008). This could however be reduced by heat, sun drying and boiling (Menon, 1982). The fibrous nature and high content of cell wall constituents (neutral and acidic detergent fibre and lignin) suggest that cacao bean shells can be more suitable in feeding ruminants than monogastrics (Flachowsky *et al.*, 1990).

According to Ashun (1975), CBS has been a useful ingredient in cattle feeding (for meat or milk production) before the 1960s. Hence it can be used as roughage in ruminant diet (as shown in sheep and dairy cattle) up to 5% of dry matter intake with appreciably high rumen degradation and digestion (Flachowsky *et al.,* 1990). Although there are limited information on the use of

CBS in fish diet, however, Bamba *et al*. (2014) reported a significant improvement in growth performance of Nile tilapia fish fed diet with 23% inclusion of CBS. On the other hand, Olubamiwa and co (2001) in their study showed that higher dietary replacement of maize by CBS beyond 10% resulted in lower performance of layers. Also a similar report showed that CBS was used in the diet of rabbit up to 10% without any adverse effect (Ayinde *et al*., 2010).

2.5.4 Cocoa bean meal / Cocoa meal / Cocoa bean cake (CBM/CM/CBC)

During processing of cocoa, cocoa beans are roasted, fermented, and ground into a mixture that can be pressed to separate out the cocoa oils. The remaining residue is known as the press cake, and this is ground to form cocoa powder (Gartrell and Roe, 2012). When there is excess cocoa production, surpluses may be sold for livestock feeding under the name of cocoa bean meal hereafter sometimes referred to as cocoa meal or cocoa bean cake (EFSA, 2008). Cocoa meal may also be prepared from discarded cocoa beans, press cake of cocoa beans, or residues from cocoa factories. The composition of the meal is greatly dependent on the amount of incorporated shell fragments in the meal and the degree of oil extraction (EFSA, 2008). The proximate composition of the cocoa meal as reported are summarized in Table 9. A major limiting factor to the use of cocoa meal in animal diet include its high content of theobromine (20-33 g/kg) and caffeine (1-4g/kg). This could however be reduced or extracted by treatments with sodium hydroxide or warm water of varying temperatures (above 60^oC) thus improving its usefulness in animal production (Adegbola and Omole, 1973).

There is however limited information on the use of CBM in poultry, livestock and fishery production.

2.6 REPRODUCTIVE STRUCTURES OF THE CATFISH: THE GONADS

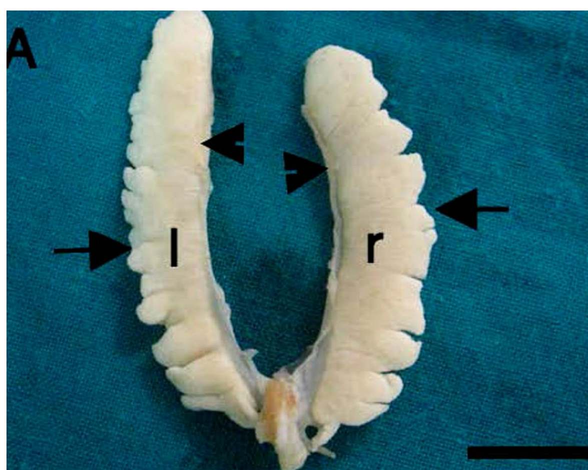
2.6.1 The Testes

C. gariepinus shares the basic testicular structure of other fish (Meisner *et al*., 2000), avian (Friedlander *et al*., 1992), and mammalian species (Ahmed *et al*., 2012). It consists of a pair of elongated and serated structure, dorsally located in the body cavity with the left often being longer than the right (Loir *et al*., 1989; Van Dyk and Pieterse, 2008). The fish testis parenchyma is composed of two main compartments: the seminiferous lobules and the interstitial connective tissues which separates the lobules and contains testosterone secreting leydig cells. (Schulz *et al*., 2010; Yasser *et al*., 2013). The connective tissue septae which separate the lobules forms a continuum with the covering tunica albuginea (Meisner *et al*., 2000). The seminiferous tubules

are made up of the sertoli cells and the spermatogenic cells or the germ cells (Yasser *et al.*, 2013).

The sertoli cells are elongated with a light nucleolus and their proliferation has been mentioned as the major factor determining testicular size in fish (Schulz *et al.*, 2010). The periphery of the catfish testis has a spermatic duct lined with cuboidal and columnar epithelium which is found to be filled with spermatozoa in some areas but empty in some other regions (Yasser *et al.*, 2013).

Plate 1: Gross structure of the testes of a mature male *C. gariepinus*



Source: Yasser ***et al.* (2013)**

The spermatogenic cells which include primary and secondary spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa are known to be present along the entire length of the testis in most catfish families. Hence, spermatogenesis occurred along the entire cranial to caudal part of the testis (Chaves-Pozo *et al.*, 2005) and this has been termed unrestrictive testis (Grier, 1981). Spermatogenic cells of equal maturity are found in groups within cysts made by the processes of the sertoli cells (Dyk and Pieterse, 2008; Schulz *et al.*, 2010). These spermatogenic cells have been reported to progressively decrease in size as they transform into spermatozoa with histomorphological variations with which each cell type can be identified (Yasser *et al.*, 2013). The spermatozoa is reported to be mono-flagellated (Matos *et al.*, 2002) and acrosome-less (Yasser *et al.*, 2013) which is common in teleosts which engage in external fertilization (Lopez *et al.*, 2004). Mature spermatozoa are released into the spermatic ducts and on contraction of muscular fibers of this spermatic ducts transport of sperm through the genital papillae is facilitated (Yasser *et al.*, 2013). According to Garg and Sundararaj (1985),

catfish are naturally seasonal breeders with the level of testicular activity and spermatogenic rate being greatly influenced by photoperiod and temperature changes.

2.6.2 Ovary

The ovaries of *C. gariepinus* are paired elongated organs which lie dorsally in the body cavity (Haruna, 2003). Each ovary consists of an outer membrane with lamellae penetrating the central lumen. The lamellae contain oogonia and oocytes in follicles at different stages of development. Each ovary extends posteriorly into a distinct oviduct (Coche and Edwards, 1990). The two oviducts fuse at their end and open into a urogenital papilla, situated just behind the anus. Mature females have very large ovaries which fill the body cavity and may constitute up to 25ó30% of their total body weight (Coche and Edwards, 1990). This means that a female of 1kg will have about 250 ó 300g of ovary. Similarly, the eggs of a "ripe" female make up 15-20% of the body weight. In nature the ovaries of a mature population usually represent from 7 to 12% of the total body weight of females (Micha, 1973; Bruton, 1979). Prior to maturity, a fishøs ovary undergoes marked changes (morphologically and histologically) until it ripes and these changes are influenced by some factors both internal (pituitary and hormone) and external factors (such as food and environmental conditions) (Gupta and Gupta, 2006).

2.6.3 Gonadal development and maturation of African Catfish

On introduction of sharptooth catfish to aquaculture, a careful study and understanding of its biological processes including its reproductive performance and gonadal morphology, anatomy, histology and development are key factors to consider for proper management and the improvement of its culture (Yasser *et al*., 2013; Cek and Yilmaz, 2007). As seen in other teleost and mammals, the reproductive system of the catfish is under the control of the hypothalamus-hypophyseal-gonadal axis and their associated hormones and chemical substances (Yousefian and Mousari, 2011).

As has been reported, the reproductive performance of *C. gariepinus* in captivity or laboratory conditions is greatly different from those of their natural environment (Ritcher *et al*., 1995). The reproductive biology of *C. gariepinus* have been studied under laboratory conditions (Cek and Yilmaz, 2007) and under natural condition (Yalcin *et al*., 2001; Yasser *et al*., 2013). Besides the study conditions, region of study also influences reported performance parameters of African catfish with Nigerian studies reporting varying results from those reported in several regions of the world (Saka and Adeyemo, 2015).

The development of the gonads can be studied and followed through both macroscopically and microscopically. The macroscopic evaluation involves using eye judgments to identify distinctive features and variations in the gonads while the microscopic determinations consist of histological studies using chemicals and laboratory procedures to prepare the tissues for viewing under compound light microscope (Cevaco *et al*., 1997; Saka and Adeyemo, 2015). Turkish *C. gariepinus* was reported to attain sexual maturity about a year after hatching under laboratory conditions with initial detection of eggs at 9 months of age and at 123 grams body weight for the females (Cek and Yilmaz, 2007). Similar report of 113 grams body weight for the female maturing at 1 year was also made in the wild (Yalcin *et al*., 2001). There have been conflicting reports as to which size and age, at maturity should be depended on. While Schulz *et al*. (1994) attributed the key determinant of maturity to age alone, Cek and Erdal (2007) maintained it is size although age has a contributive role. Gonadal development of african catfish has been studied extensively and reported (Cek and Yilmaz, 2007; Ekanem *et al*., 2012; Yusuf *et al*., 2013; Saka and Adeyemo, 2015).

According to Cez and Yilmaz (2007), it was not possible to distinguish the male and female Turkish *C. gariepinus* until 5 months as the gonads appear as a thin, translucent, creamy white, ribbon-like structure attached to the dorso-lateral lining of the peritoneal cavity. On the other hand, Saka and Adeyemo (2015) reported that the gonads of the Nigerian female *C. gariepinus* was activated and became differentiated from the male at 3 months and hence, the sexes could be distinguished by this age if the fish is dissected.

2.6.3.1 Ovarian development

The development of the ovary follows a progressive pattern and is classified in *C. gariepinus* as a group-synchronous type. The appearance, colour, texture, presence and size of the oocytes and eggs. Starting from the 3rd month the ovary can be differentiated from the testes when the fish is dissected (Saka and Adeyemo, 2015). It is seen attached to the dorsal-lateral lining of the peritoneal cavity. Several stages of ovarian development have been described by various researchers using varrying criteria for classification. Some classified ovary development into eight stages (Utoh *et al*., 2003; Van der Molen and Matallanas; 2004; Gupta and Gupta, 2006; Solomon and Ramnarine, 2007), seven stages (De Martini *et al*., 2000; Adebiyi *et al*., 2011; Cek *et al*., 2001), six stages (Murua *et al*., 2003; Chelemal *et al*., 2009; Shinkafi and Daneji, 2011), five stages (Abascal and Medina, 2005; Morris *et al*., 2011) and four stages (Ravaglia and Maggese, 2002; De Lestang *et al*., 2003; Kumar *et al*., 2003; Nejedli *et al*., 2004; Koc *et al*., 2008). As described by Owiti and Dadzie (1989) and Saka and Adeyemo (2015),

there are six chronological stages of ovarian development/oogenesis for *C. gariepinus*. The striking difference in the different classifications is that while spawning commenced in stage 5 according to Owti and Dadzie (1989), it was shown in stage 4 by Saka and Adeyemo (2015). The histological descriptions are made based on the presence and prominence of the various oocyte developmental stages. The different oocytes stages ranging from oogonia to the yolk granule stage found in catfish is presented in Table 11.

At stage 4 of ovarian development, the protein needed for the yolk formation is synthesized in the liver and transported to the ovary. The oocytes present in the ovary at this stage are called õripe eggsö and the oocytes remain in this stage until stimulated by environmental factors (such as rainfall, rise of water level or hormonal injections) to be ovulated (Owiti and Dadzie, 1989). This transition will take place once the water temperature is 20 ó 22°C or higher. Although different authors have classified this stage under different phase of development and have also reported the attainment of this stage at different ages and seasons/ months. After ovulation of the ripe egg, the majority of the oocytes found in the ovary are composed of stage 1 oocytes, the cycle is repeated and after approximately six weeks a new batch of "ripe eggs" is ready for ovulation (De Graaf and Jannsen, 1996).

Table 11: Stages of ovarian development using macroscopic and microscopic descriptions

Stages	Macroscopic/ Physical and histological (Histological descriptions: Mollah, 1986) Description	
	1	**2**
Stage 1 (ST1)	**Immature virgin:** The ovary is colourless to translucent brown, lanceolate and lobular in appearance, occupying the posterior quarter of the body cavity. It can be distinguished from the testis in fish larger than 10 cm due to its smoothness in contrast to the serrated edges of the testis. **Histological**: ovary contains oogonia and chromatin nucleolar oocytes.	Observable at three months of age. The ovaries were attached to the dorsal-lateral lining of the peritoneal cavity. Growing like a tube, hollow as a thin ribbon-like structure, creamy-white and translucent. The edges are smooth and the ovary looks so much like the testes. It is quite difficult to differentiate between the ovary and the testes at this stage.
Stage 2 (ST2)	**Developing virgin:** The ovary is translucent to brown in colour and occupies about one third of the length of the peritoneal cavity. Individual oocytes are visible with the naked eye as tiny specks. **Histological**: ovary contains a large reserve of early and late perinucleolar oocytes.	This is the maturing stage. The ovaries had smooth edges, they were reddish-brown and granular in appearance. By the end of stage 2, the ovaries are completely filled up with clearly visible oocytes of different sizes which will be whitish.
Stage 3 (ST3)	**Maturing:** The ovary is opaque, brownish-green in colour, occupying about one half the length of the ventral cavity. Eggs are visible as yellowish-green or brownish-yellow granules. Blood capillaries are visible around the ovary. **Histological**: yolk-vesicle oocytes are predominant	Occurs at the 4th month and referred to as maturation stage. In the stage, the ovaries occupy a significant portion of the abdominal cavity. They are big and seen as visible greenish oocytes with a well-developed vascularization. The ovaries are usually matt-green at the onset of this stage and clear green at the peak of this stage. However, the oocytes are still included in the ovarian lamellae, and the ovary lumen was empty. These ovaries characterize fully vitellogenic females, close to the spawning period.
Stage 4 (ST4)	**Ripe/ Mature:** Exogenous vitello genesis. Ovaries are large (occupying more than ¾ of the peritoneal cavity), opaque, and brown-green in colour. Egg contain yolks clearly visible to the naked eye. **Histological**: Early yolk granule oocytes predominate, although some yolk vesicle oocytes are also present. During this phase, yolk formation in the oocyte increases.	Occurs at 5 months and represent the stage of maximal ovarian development. The ovaries transparent clear green and contains greenish oocytes. Almost identical to stage 3 ovaries, but the oocytes are partially ovulated (free in the ovarian cavity = ovules) and can be expelled with a gentle pressure on the fish flanks. This is an ephemeral stage just before the actual spawning event. Ovaries at this stage are said to be ripe.
Stage 5 (ST5)	**Spawning:** Eggs are flat, translucent and easily extruded on slight pressure or just by handling the fish. **Histological**: ovary contains mainly late yolk-granule oocytes.	No clear physical descriptions. But histologically it is shown that oocytes in this stage had the biggest diameter.
Stage 6 (ST6)	**Spent**: Ovary is flaccid, flabby with thick whitish tough walls. Genital aperture of female looks inflamed. Some translucent and opaque (residual) eggs are visible to the naked eye. **Histological**: ovary contains predominantly degenerating oocytes and some oogonia.	NA

Histological descriptions: Mollah, 1986; 1: Owiti and Dadzie (1989); 2: Saka and Adeyemo (2015)

2.6.3.2 Developmental Stages of the Oocytes

Developing eggs pass through a series of cytological stages collectively referred to as oogenesis starting from the mother cells or oogonia until the maximal ripening stage (Mahmoud *et al*., 2009). A detailed description of the oocyte development with associated histomorphometric values was given by Cek and Yilmaz (2007). According to Cek and Yilmaz (2007), the phases of oocyte development is traditionally divided into 3 stages: primary growth phase (PGP); secondary growth phase (SGP); and the maturation phase which includes the hydration phase (MHP). They classified the process of oogenesis on the basis of the appearance of nuclei and nucleoli, and on the distribution of cytoplasmic inclusions. Various authors (Rastogi and Saxena, 1968; Mollah, 1986) classified the changes exhibited in the nucleus and cytoplasm and the formation of yolk into nine separate stages in order to categorize the developing oocyte of catfish (*Clarias* macrocephalus). This includes:

I. **Oogonia**: Usually found singly or in small nests in post-ovulatory fish. Even during the post-ovulatory months only a small number of oogonial divisions were observed, suggesting a rapid transformation of oogonia into oocytes. Oogonial cysts were rarely seen in maturing ovaries with yolky oocytes. Oogonia were -8-12 µm in diameter, each with a single nucleus very little cytoplasm.

II. **Chromatin-nucleolar stage (early and late):** Oocytes in this stage were distinguished from the oogonia by the presence of distinct chromosomes in various stages of meiotic prophase. Oocyte diameter varies from 10 to 20µm.

III. **Early perinucleolar stage:** This stage can be easily identified by the peripheral arrangement of a large number of small nucleoli on the inner side of the nuclear membrane. They have varying shapes possibly influenced by the imposed stress of the expanding oocytes around them. Oocyte diameter varies from 25 to 50µm

IV. **Late perinucleolar stage:** In this stage, the shape of the oocytes become more regular with a diameter ranging from 30 to 250µm. Also the nucleolus is enlarged in this stage.

V. **Yolk vesicle stage (early and late):** In this stage, there is the formation of yolk vesicles in the periphery of the ooplasm. They initially form as a single row which appear colourless when the slides were stained with haematoxylin and eosin. These yolk vesicles developed initially as minute bodies but gradually increased in size and number to form several irregular peripheral rows. Also the nucleoli is usually present at the periphery of the nucleus, but, can also appeared elsewhere in the nucleus in some cases. The zona radiata starts to develop and the follicular layers also well developed with thecal and

granulosa cells. The diameter of oocytes at this stage varied from 200 to 500 µm with a nuclear diameter of 60 to 175 µm.

VI. **Early yolk granule stage:** This stage which involves the formation of yolk granules appears in the final stage of vitellogenesis and oocyte development. Yolk granules formed only in oocytes with fully developed yolk vesicles. The yolk granules first appeared close to the zona radiate and at a later stage the whole oocyte was filled with yolk granules. Oocytes at this stage were generally between 500 and 800 µm in diameter.

VII. **Late yolk granule stage**: The diameter of the oocytes increased simultaneously with the advancement of yolk granule stage. In some cases the yolk granules appeared to coalesce to form larger, tightly packed granules. The oocyte at this stage might reach a diameter as big as 1060 µm, whereas the individual granules were about 25 µm or less in diameter.

The summary of these stages as reported (Owiti and Dadzie, 1989; Cek and Yilmaz, 2007) are presented in Table 12.

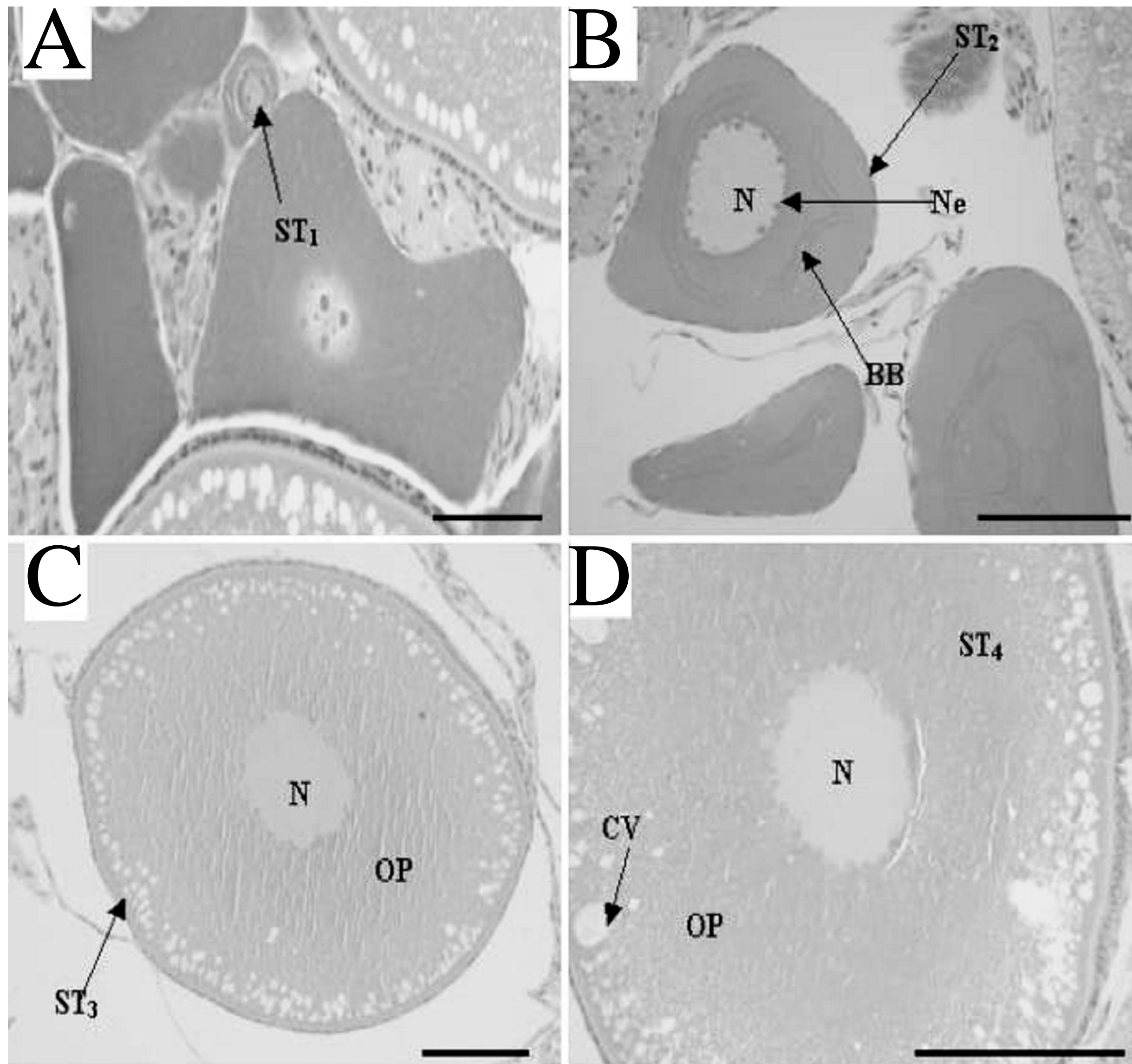
As earlier stated, the histologically characterization of oocyte development described for *C. gariepinus* is divided into Primary growth phase, Secondary growth phase, and maturation. The maturation phase included hydration and stage 6 oocytes. As also reported in other teleost, oogonia of *C. gariepinus* proliferated and turned into primary oocytes, which subsequently grew within follicles, formed cortical alveoli, commenced vitellogenesis, underwent maturation and finally ovulated (Saka and Adeyemo, 2015). According to Cek and Yilmaz (2007), the ovaries of the fish were in the resting stage for the first 6 months of age (Until March) in their study, during which age they contained mainly oocytes at the chromatin and perinucleolar stage. SGP began in the 6th month and ended by the 8th month (Cek and Yilmaz, 2007). Although Saka and Adeyemo (2015) reported this stage at 4 months of age. Mainly 2 oocyte stages were distinguished in the ovaries of *C. gariepinus* namely, vesicle formation and exogenous yolk formation (Cek and Yilmaz, 2007). The latter differed from the former by the presence of a yolk precursor protein, vitellogenin. Exogenous yolk formation was characterised by the presence of yolk granules. The yolk granules stained pink with haematoxylin and eosin and were first detected close to the microvilli of the oocyte membranes and later in the ooplasm. As development progressed, these yolk granules coalesced to form larger yolk globules. At the same time they were pushed towards the centre of the oocytes. The largest oocytes were stage 6 oocytes in mature ovaries (Owiti and Dadzie, 1989). They were completely yolk-filled structures.

Table 12: Phases of oocyte development in *Clarias gariepinus*, based on histological criteria

Phase	Stages	Histological Description	
References		**Cek and Yilmaz (2007)**	**Owiti and Dadzie (1989)**
Primary growth phase (PGP)	Stage 1: Chromatin nucleolar stage	This stage was characterized by a large nucleus in the central position, surrounded by little cytoplasm. At this stage the diameter of oocytes was 3.17 ± 0.19 µm.	Pre-vitello genesis or primary oocytes: Nests of oogonia, numerous early, darker staining and few advanced lighter-staining, pre-vitellogeneic (yolkless) oocytes visible. Oocytes are small (7-10 micron)
	Stage 2: Perinucleolar stage	Nucleus increased in size and nucleolus increased in number. Balbiani bodies appeared in the cytoplasm. At the end of this stage balbiani bodies were distributed all over the cytoplasm. Oocyte diameter ranged from 5.85 ± 0.87 to 6.75 ± 0.35µm.	Pre-vitello genesis or primary oocytes: The oocytes do not yet contain yolk. The size and number of primary oocytes increases (7-10 micron).
Secondary growth phase (SGP)	Stage 3	Cortical vesicles were detected for the first time. These were usually spherical structures that appeared at random at various depths in the ooplasm. They provided the first evidence for initiation of the secondary growth phase and appeared usually as empty unstained vacuoles. The diameter of the stage 3 oocytes was 14.71± 2.12 µm.	Endogenous vitello genesis: Oocytes in early vitellogenic phase can be observed. The oocyte yolk (the future reserve/ feed for the hatched larvae) is formed.
	Stage 4	The nucleus consisted of many nucleoli and continued to enlarge, becoming very irregular in shape. The zona radiata was more conspicuous. The process of vacuolisation was completed by the formation of 2 rows of vacuoles. Stage 4 oocytes were 29.25 ± 0.88 µm in diameter.	Exogenous vitello genesis: The oocyte increases to its final size of 1000-1200 m. A large nucleus (200 m) is clearly visible with signs of migration towards the periphery of the oocyte. The oocytes remain at this stage until environmental factors (rainfall or a hormonal injection) stimulate ovulation.
	Stage 5	Yolk granules were first detected only between vacuoles and later in the cytoplasm free from them. The nucleus showed a significant number of projections into the cytoplasm. The development of the egg shell was completed with the zona radiata and vitellin membrane. The diameter of stage 5 oocytes was 64.80 ± 3.41 µm.	Large eggs contain coarse granules throughout the cytoplasm. Germinal vesicles start to migrate to the animal pole, where they remain.
Maturation and hydration phase (MHP)	Stage 6	This stage was distinguished by migration of the nucleus to the animal pole, where it remained, but the nuclear membrane disintegrated. The nucleus was smaller in size. The nucleoli were smaller than the previous stage, and hardly distinguishable in the nucleus. The layers of oocytes were thinner than those of stage 5 oocytes. However, during oocyte maturation and ovulation, the zona radiata increased rapidly in size. After the germinal vesicle breakdown, the oocytes ovulated into the ovarian lumen and the post ovulatory follicle remained in the ovary. Oocyte diameter at these stages ranged from 105 ±1.97 to 125 ± 5.95 µm.	Large numbers of ruptured follicles visible in ovary. Some yolky eggs undergoing atresia as well as presence of early yolky oocytes, non-yolky oocytes and oogonia.

The micrograpical representation of the various oocytes stages is shown in figure 5 below.

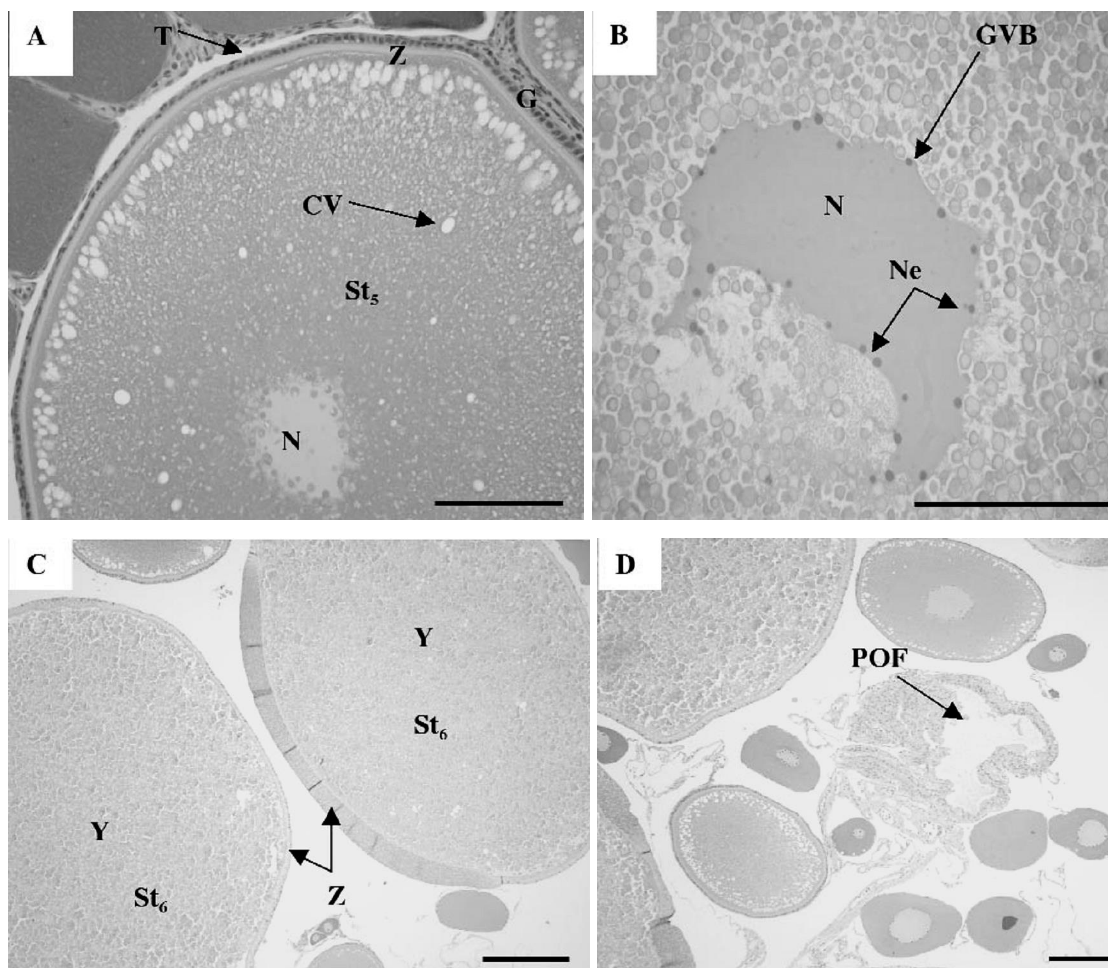
Plate 2: Stages 1, 2, 3, and 4 of oocyte development of *C. gariepinus*



Sections of African catfish ovaries in different stages of oogenesis. A: Oocytes at primary growth phase (St1: stage 1 oocytes). B: Oocytes at primary growth phase (BB: Balbiani bodies; St2: stage 2 oocytes). C: Oocytes at secondary growth phase (CV: cortical vesicles; St3: stage 3 oocytes). D: Oocytes at secondary growth phase (St4: stage 4 oocytes; N: nucleus; Ne: nucleoli; OP: ooplasm; CV: cytoplasmic vesicle). All scale bars = 200 µm.

During the breeding season, from May to July (8 to 10 months of age), ovaries were in the post-vitellogenic or post-ovulation stage (Cek and Yilmaz, 2007). Contrary to these authors, Saka and Adeyemo (2015) reported the occurrence of stage 4 oocytes at 5 months of age. According to them (Saka and Adeyemo, 2015), stage 4 represents the ephemeral stage before the actual spawning event. This is the stage of maximum development and the ovaries at this stage is said to be ripe. However, a full-grown ovary sometimes was found to enter a phase of regression when environmental conditions were not suitable (Cek and Yilmaz, 2007).

Plate 3: stages 5 and 6 of oocyte development and post ovulatory follicle of *C. gariepinus*



Sections of African catfish ovaries in different stages of oogenesis. A: Oocytes at stage 5 (St5: stage 5 oocytes). B: Oocytes at maturation phase (arrow shows germinal vesicle breakdown; GVB), (St6: stage 6 oocytes). C: Zona radiata underwent changes during oocyte maturation and ovulation. D: Arrow shows post-ovulatory follicle (POF). N: nucleus; Ne: nucleoli; Y: yolk; CV: cytoplasmic vesicle; T: theca; Z: zona radiata; G: granulosa. All scale bars = 225 µm.

2.6.4 TESTICULAR DEVELOPMENT

The general morphology of the testes have been described earlier. The *C. gariepinus* have been reported to mature six months after hatching (Schulz *et al*., 1994; Yalcin *et al*., 2001; Saka *et al*., 2015). However, when the development of the male *C. gariepinus* is followed to maturity, four distinct stages have been identified on gross morphological basis. According to Saka *et al*. (2015), these stages include:

- **Stage I:** Testes are generally small, silvery or translucent filaments, thinner and longer than stage I ovaries. They have rounded edges which appeared like a tube. This was observed at the onset of gonadal development (12 weeks post hatch).

- **Stage II:** Stage II Testes are characterized by whitish to pinkish, relatively large and turgescent testes of circular section. However, semen was not present within the sperm duct and even when squeezed, there was no expression of milt.
- **Stage III**: Testes are larger, fuller and well-developed. The entire gonad or only some areas had a whitish color. A slight pressure of the testes surface makes milt flow out of the sperm duct, which is a definite indication of the stage III testes. This is manifested at about five months.
- **Stage IV**: Testes are still almost as large as stage III testes, but they were flaccid and empty. This stage is indicative of a male just after spermiation (spent stage).

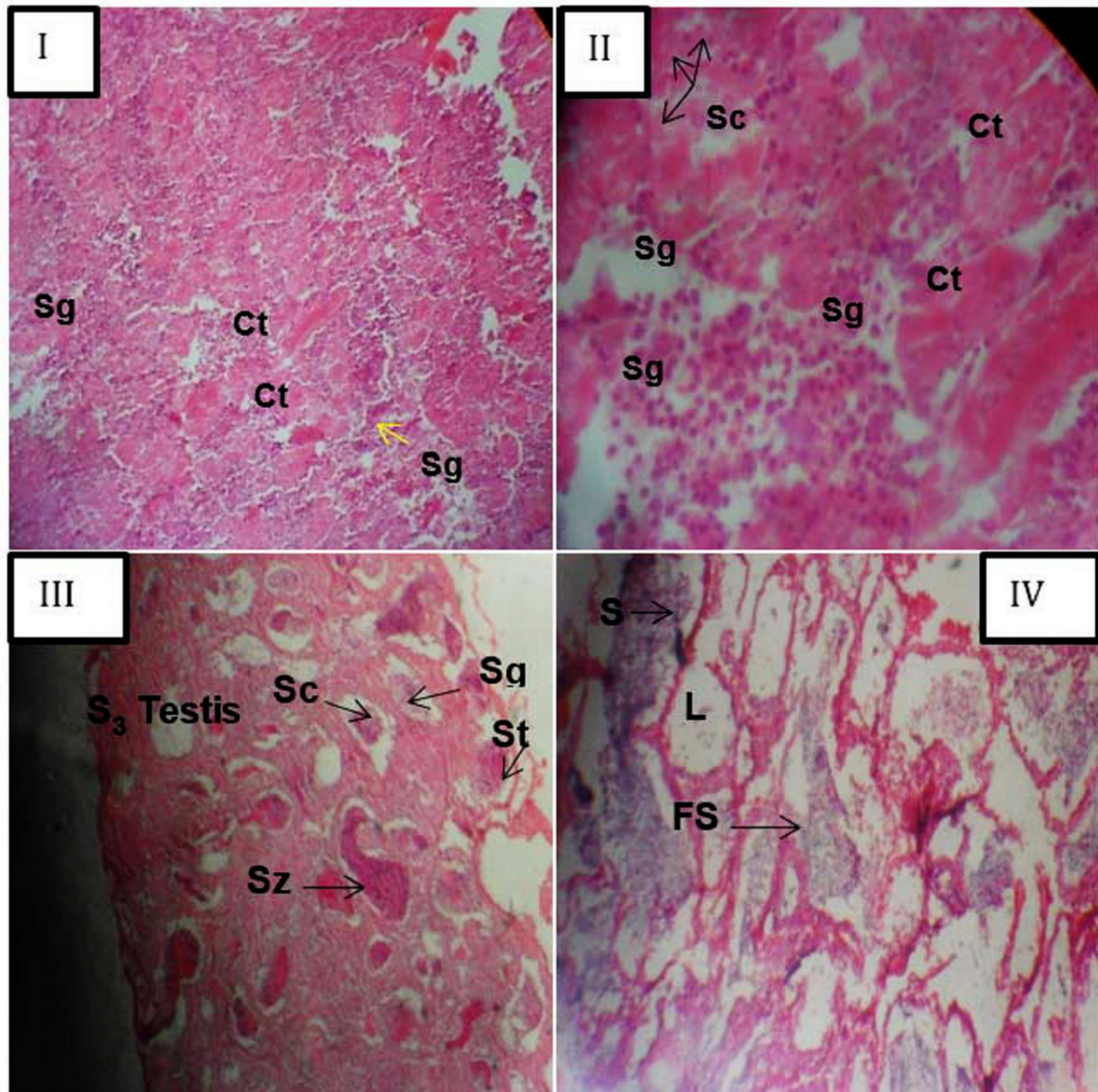
The testes of the *C. gariepinus* as have been described before is of the unrestrictive type (Anastomosing tubular type) where spermatogonia were distributed throughout the testes (Cevaco, 2005; Cek and Yilmaz, 2007). During the pubertal period, the primary and secondary spermatocytes were reported to be motionless (Schulz *et al*., 1994; Cevaco *et al*., 1997). As reported by Wu *et al*. (2001), and Viveiros *et al*. (2002), Cek and Yilmaz (2007), milt cannot be obtained from the posterior part of the testes of a mature male (suggesting that this region could be non-germinal) when dissected, whereas milt was readily obtained from the anterior end which contains spermatogenic cells. The testicular development on histomorphological basis have been placed in several stages with some variations among different authors. Whereas Saka *et al*. (2015) reported 4 developmental stages, Cek and Yilmaz (2007) reported 5. This is presented in table 13.

Table 13: Testicular development stages of the *C. gariepinus* testes based on Histomorphology

Stages	Histological descriptions	
Reference	**Cek and Yilmaz (2007)**	**Saka *et al*. (2015)**
Stage 1	Testes contained spermatogonia only.	Testes were characterized by the presence of spermatogonia nests embedded in abundant connective tissue. Spermatogonia were visible but there were more somatic cells than spermatogonia, primary spermatocytes were also observed. This stage is observed at week 12 post hatching.
Stage 2	Testes showed spermatogonia, primary spermatocytes, and meiotic germ cells.	Had more spermatogonia, somatic cells and spermatocytes and less connective tissue. Lobular structure/arrangement of the seminiferous tubules begins to appear at this stage.
Stage 3	Testes contained spermatogonia, primary and secondary spermatocytes, and meiotic germ cells.	All stages of spermatogenesis were present in well-developed seminiferous tubules. Spermatids and spermatozoa were darkly-stained. Spermatids were seen and were smaller than the spermatocytes, irregular in shape and more basophilic. Maturation was evidenced by the prevalence of spermatozoa which was seen to be strongly basophilic. This stage is observed between 4 to 5 months post hatch.
Stage 4	Testes contained spermatids, but no spermatozoa.	Testes had a predominance of mature spermatozoa and is characterized by the emptying of spermatozoa into the lumen to create free spermatozoa.
Stage 5	all germ cell stages, including spermatozoa, were present	NA

The histomorpohology of the stages of gonadal development is shown in plate 4.

Plate 4: Histomorphology of the different developmental stages of the *C. gariepinus* testes.



Source: Saka *et al.* (2015). I: Stage 1 testes showing proliferation of spermatogonia and connective tissue; II: Stage 2 testes showing an increased number of spermatogonia and spermatocytes (Sg: spermatogonia, Sc: spermatocytes and Ct: Connective tissues); III: Stage 3 testes, different stages of spermatogenesis may be identified by the absorption of the stains (Sc: Spermatocytes, Sg: Spermatogonia, St: Spermatids, Sz: Spermatozoa); IV: Stage 4 testes showing the emptying of the spermatozoa into the lumen (S: Spermatozoa, FS: Free Spermatozoa, L: Lumen).

2.6.5 PHASES OF SPERMATOGENESIS DURING CATFISH DEVELOPMENT

The paired testes of adult *C. gariepinus* are composed of numerous finger-like projections, which extended from their respective sagittal axes. The bilateral testes fused together in their posterior region to form the seminal vesicle. The tubules of the seminal vesicle contained non-germinal epithelia and are shorter and thinner than those of the anterior region (Cek and Yilmaz, 2007). Formation of spermatozoa in the *C. gariepinus* was divided into 5 stages in samples taken from the anterior part of the gonads

Table 14: Phases of spermatogenesis in *Clarias gariepinus*, based on histological criteria

Phases	**Description**
Spermatogonia (ST1)	The spermatogonia are the largest cells in the germinal tissue of the *C. gariepinus* testes. The nucleolus is large and lay close to the centre of the nucleus. These cells divide mitotically and form primary spermatocytes. Somatic cells around the spermatogonial cells are clearly visible.
Primary Spermatocytes (ST2)	The primary spermatocytes are smaller than the spermatogonial cells and their daughter cells. They are spherical and presented as small groups (nests). Primary spermatocytes divide meiotically to produce secondary spermatocytes. They had no visible nuclear membrane and the chromatin material occupy most of the cell.
Secondary Spermatocytes (ST3)	Secondary spermatocytes are morphologically similar to primary spermatocytes, though somewhat smaller and more basophilic. Their nucleolus is not clearly detected. The differentiating germ cells continue to have a close morphological relationship with the cyst cells, which form the cytoplasmic processes extending between the spermatogonial cells.
Spermatids (ST4)	Secondary spermatocytes continue meiotic division and produce spermatids. The spermatids are smaller than the secondary spermatocytes, irregular in shape, and very strongly basophilic.
Spermatozoa (ST5)	Transformation of spermatids into mature spermatozoa consists of a reorganization of the nucleus and cytoplasm, together with the development of a flagellum. No cell division was visible.

Source: Cek and Yilmaz (2007)

Phases of spermatogenesis were distinguishable on the basis of their characteristic nuclear and cytoplasmic morphologies. All stages of spermatogenesis, including ruptured spermatozoa, were detected in the sperm ducts. Lobules containing numerous spermatocytes, from early stages (spermatogonia) to complete spermatogenesis (spermatocytes, spermatids and spermatozoa), were observed. However, the posterior part of *C. gariepinus* gonads contains mostly spermatogonia and spermatocytes although free spermatozoa were occasionally recorded (Cek

and Yilmaz, 2007). The anterior part of the testes contained mostly free spermatozoa. At the age of 6 months they were clustered with their heads attached to the lobules, but subsequently (at the age of 8 months) the lobular walls break down, and sperm become unattached and lay free in the lumen (Cek and Yilmaz, 2007). In the resting period (extending from August to May), the testes contained only spermatogonia. In May, June, and July, the anterior part of the testes contained spermatids and ruptured cysts with its released spermatozoa.

2.7 FACTORS AFFECTING GONADAL DEVELOPMENT

As earlier stated, attainment of sexual maturity and development is a function of age and size. The development from stage 1 to stage 4 is related to temperature (and of course age of first maturation) while the development from stage 4 to stage 5 is triggered by environmental stimuli or can be provoked by hormonal injections. Several factors have been reported to affect gonadal development of catfish and teleost fish in general. These factors include:

2.7.1 Season

C. gariepinus has been reported to show a seasonal gonadal maturation which is usually associated with the rainy season. The maturation processes of *C. gariepinus* are largely controlled by annual changes in water temperature and photoperiodicity and the final triggering of spawning is caused by a raise in water level due to rainfall (de Graaf *et al*., 1995).

An example of maturation and spawning of *C. gariepinus* in Lake Victoria (Kenya) as reported by (De Graaf and Jannsen, 1996) shows that reproductive activities commences in March just after the onset of the first heavy rains as was indicated by the decrease in the Gonado Somatic Index1 (G.S.I.). Natural reproduction is completed in July and the G.S.I. remains low till November, thereafter oocytes maturation gradually commences, becoming ripe again in March.

2.7.2 Temperature

According to timothy (2010), development and maturation of the gonads require a certain õtemperature rangeö, usually quantified as hour grades or day-grades, i.e the number of hours or days at which a given temperature needed to accomplish gonadal maturation occurred. The optimum temperature of 25^{0}C with a minimal fluctuation is a pre-requisite for qualitatively and quantitatively adequate gonadal development year round (Richter *et al* 1982). The development from stage 4 to 5 will usually take place once the water temperature is 20-22°C or higher (De Graaf and Jannsen, 1996). The oocyte development has been reported to decreases once the temperature drops below 22°C. After ovulation of the "ripe eggs" the majority of the oocytes found in the ovary consists again of stage 1 oocytes, the cycle is repeated and after

approximately six weeks a new batch of "ripe eggs" is ready for ovulation. As reported by De Graaf and Jannsen (1996), the eggs which make up 15 ó 20% of the female body weight decreases to 5 % when the temperature drops below 22 ^{0}C. On the other hand, temperature was not found by this authors to affect the availability of sperm in the males.

2.7.3 Feeding levels

Exogenous nutrition of catfish Broodstock has been reported to provide the indispensable nutrients necessary for the gonadal development of females and the performance of the seed products (Gunasekera *et al*., 1997; Hirofumi, 2009). Proper health of the fish can be assumed by adequate feeding of the brood stock for a sufficient time before spawning occurs (Richter *et al* 1982). Early maturation in male *C. gariepinus* have been achieved by genetic selection and better nutrition, suggesting a correlation between maturation and growth (Le Bail, 1996).

Vitamins are important in the process of sexual maturation as they have been reported to play a vital role in the biosynthesis processes of gonadal steroid hormones (Hirofumin, 2009). Deficiencies of vitamin A or carotenoids in the female *Clarias lazara* decreases the chances of survival of the eggs and larvae (Shehadeh, 1975). Vitamin C is required for the biosynthesis of collagen in connective tissue and for normal development while vitamin E increases egg production (Takeuchi, 1997).

During maturation of the gonads (ovary and testis), there is an accumulation of non-saturated fatty acids in the ovaries and increase in the level of protein and dry matter. Shehadh (1975) reported that fat accumulation reached a peak in *Clarias lazera* just before their gonads attained full development. Fat reserves probably provide readily utilizable energy for the rapid production of gonadal materials. Furthermore, Watanabe (1993) and Yanes-Roca *et al*. (2009) reported that lipids have a role as membrane constituent and energy reserves in fish embryos. They further maintained that n-3 highly unsaturated fatty acids (n-3 HUFA), in particular docosahexaenonic acid (DHA), are essential for larval development. The fatty acid composition of eggs is directly affected by the fatty acid composition of the broodstock (Mourente and Odriozola 1990). According to Hirofumi (2009), dietary protein is necessary for proper growth performance of broodstock fishes and an average of 45% crude protein is optimal.

2.7.4 Age and size

Full maturation of *C. gariepinus* was found to be vary according to several authors. While Cek and Yilmaz (2007) reported this to be 1 year other studies (Schulz *et al*., 1994; Yalcin *et al*., 2001; Saka and Adeyemo, 2015; Saka *et al*., 2015) reported full maturity at 6 months of age. Schulz *et al*. (1994) stated that sexual maturity is related to age in *C. gariepinus*; however, Cek

and Yilmaz (2007) and Saka *et al*. (2015) showed that it was more determined by size rather than age, although age cannot be totally excluded in the determination of puberty, especially since the age at puberty appears to decrease with increased size.

2.7.5 Others factors

Other factors affecting gonadal development of African Catfish include water depth, salinity, and PH.

2.8 GONADOSOMATIC INDEX

Gonadosomatic Index (GSI) is a metric that represents the relative weight of the gonad to the fish weight and is used to estimate the development of gonad in fish (Usman *et al*., 2014). Because it is inexpensive and easy to compute (Flores *et al*., 2014), GSI has been widely used to evaluate reproduction timing (Lowerre-Barbieri *et al*., 2011) and can provide a quantitative assessment of the degree of gonadal development, the breeding season, and the reproductive cycle (Gutiérrez-Estrada *et al*., 2000). As seen in most fishes, GSI increases with the maturation of fish and become highest during the period of peak maturity and thereafter declining abruptly, when the fish is spent (Usman *et al*., 2014). Breeding or spawning period can be readily confirmed after determining the stage of maturity of the gonads (Gupta and Gupta, 2006). GSI is calculated as weight of ovary in grams by weight of fish in grams multiplied by 100 (Adebiyi *et al*., 2011). This is shown below

$$GSI = \frac{weight\ of\ gonad\ (grams)\ \times 100}{weight\ of\ fish\ (grams)}$$

Changes in GSI are mostly determined by variations in yolk concentration during different oocyte stages and thus it provides information about maturation and seasonal patterns in gonad development (Wallace and Selman, 1981; West, 1990). Reproductive studies are frequently based on quantitative indices, as gonadosomatic index values (GSI) and condition factor (K) when direct inspection of gonadal maturation is not available and these help to define reproductive cycles and possible variation in the physiological condition of species during the course of its lifespan (Kreiner *et al*., 2001; Braga, 2005). Previous studies have successfully applied various methods based on the gonadosomatic index (GSI) to improve accuracy in determining maturity stage (McQuinn, 1989; Vitale *et al*., 2006; McPherson *et al*., 2011). These studies separated immature fish from spent or recovering fish and identified the degree of maturity for females when neither histology nor macroscopic analyses were available (Flores *et*

al., 2014). McPherson *et al*. (2011) proposed a logistic multinomial model to evaluate maturity staging based only on GSI that was successfully applied to *Clupea harengus* to correct estimates from macroscopic staging, thus providing an alternative use of the GSI on the context of maturity staging.

2.9 CONDITION FACTOR (K)

The condition factor or the Fultonøs condition factor represented with õKö is another commonly used index in the study of fish biology, which provides information on the physiological state of the fish, based on the assumption that individuals of a given body length are in better condition when their mass is greater (Jones *et al*., 1999; Anene, 2005; Anwa-Udondiah and Pepple, 2011). According to Anyanwu *et al*. (2007), the K value is largely influenced by the fish, sex, season, maturity stage etc. The condition factor depends on the coefficient of allometry (b) of the weight-length equation, which reflects the growth pattern of a species (Le Cren, 1951). Fulton (1902) proposed the use of a mathematical formula for quantifying or estimating the condition of fish as.

$$K = \frac{100W}{L^3}$$

With W = whole body wet weight in grams and L = length in cm; the factor 100 is used to bring K close to unity

The role of the condition indices as stated by Stevenson and Woods (2006) is to quantify the health of individuals in a population or to tell whether a population is healthy relative to other populations. The inspection of the seasonal variation of the condition factor is also being used as a complementary parameter aimed at describing natural cycles in reproduction and feeding ecology (Braga, 1986; Lizama & Ambrósio, 2000; Anene, 2005).

CHAPTER THREE:

MATERIALS AND METHODS

3.1 RESEARCH LOCATION AND CLIMATIC DISTRIBUTION

The experiment was conducted at the Farm Operations Unit/WAAPP fish centre of the Faculty of Agriculture, University of Nigeria, Nsukka in Enugu State, South Eastern Nigeria. Nsukka lies in the derived Savannah region, and is located at the point of intersection of Longitude 7° 25^I N and latitude 6° 51^IE (Ejere and Okpara, 2010), with a maximum plateau altitude of 443m and a minimum of 207m above sea level (Ofamata, 1978). The climate is a humid tropical setting with a relative humidity range of 73.1% to 76.6% and annual rainfall range of 1680 to 1700mm (Breinholt *et al*., 1981).

3.2 DURATION OF STUDY

The experiment lasted through a period of 13weeks (3months and 1 week).

3.3 PROCUREMENT AND MANAGEMENT OF FISH

A total of 400 *C. gariepinus* post-fingerlings of 8 weeks old were purchased from a the West African Agricultural Programme, fish multiplication centre, Farm Operations, University of Nigeria, Nsukka, Enugu State and used for the study. The fish were kept together in a concrete pond for a period of 3 weeks (until they were 11 weeks old) for acclimatization and for proper sexing. After this period, the fish were randomly divided into ten large experimental basins (100 liters) with 20 fish per basin. The basins were randomly assigned to five experimental treatments. Each treatment was replicated four times with twenty fish per replicate in a randomized completely block design (RCBD). Feeding the fish with the experimental diets commenced from the 3th week of the study (11th week of age) through to the 12th week at the feeding rate of 3.5% of the total biomass of each basin as recommended by National Agricultural Extension and Research Liaison Services (NAERLS, 2013). The water was changed twice every week to ensure freshness, and minimize ammonia toxicity. Anti-stress was given to fish after every occasion of weighing and changing their water. The fish were weighed and recorded in grams. Both standard and total length measurements taken in cm before the commencement of the feeding trials and afterwards at weekly intervals. The fish were also observed for incidence of cannibalism and disease occurrences.

3.4 PROCUREMENT OF COCOA BEAN MEAL (COCOA BEAN CAKE)

The cocoa bean cake used for the study was purchase in the confectionary section of the Ogige urban market in Nsukka town. The lumps of the cocoa were collected and ground into fine powdery form for use in the diet formulation.

3.5 EXPERIMENTAL LAYOUT/DESIGN

The study was a completely randomized block design experiment. Prior to the commencement of the feeding trials, the fish were randomly divided into five treatments with 80 fish per treatment. Each of the treatment groups were further divided into four replicates containing twenty fish each. Treatment 1, 2, 3, 4, and 5 were fed diets containing cocoa bean meal at 0%, 10%, 20%, 40%, and 50% levels of inclusion. The feeding of the test diets started on the 3rd week, during which a total of 10 fish (5 males and 5 females) were randomly collected from the concrete pond, weighed and the measurements recorded before taking them to the laboratory. At the laboratory, the fish were dissected, to collect the gonads. The harvested gonads were weighed, recorded, studied, described, and snapped with an 8.0 megapixel camera phones. The gonads were then fixed in bouinøs fluid for further histological studies. Thereafter the fish were randomly assorted to their treatment groups. After 3 weeks of feeding the fish with the experimental diets, a total of 8 fish (4males and 4females) were randomly collected from each treatments and were also weighed and the measurements recorded before taking them to the laboratory for histology studies.

Table 15: The Layout of the study

(Number of fish stocked)	**Treatment**					**Total**
	T1 (Control)	**T2 (10%CBM)**	**T3 (20%CBM)**	**T4 (40%CBM)**	**T5 (50%CBM)**	
Male	**40**	**40**	**40**	**40**	**40**	**200**
Female	**40**	**40**	**40**	**40**	**40**	**200**
Total	**80**	**80**	**80**	**80**	**80**	**400**

CBM: Cocoa bean meal; 1 and 2 represents the replicates

Table 15: Sampling of the experimental fish for histology studies

Sampling	Sex	T1 (Control)	T2 (10%CBM)	T3 (20%CBM)	T4 (40%CBM)	T5 (50%CBM)	Total
Week 1	**Male**	1	1	1	1	1	**5**
	Female	1	1	1	1	1	**5**
Week 3	**Male**	4	4	4	4	4	**20**
	Female	4	4	4	4	4	**20**
Week 5	**Male**	4	4	4	4	4	**20**
	Female	4	4	4	4	4	**20**
Week 7	**Male**	4	4	4	4	4	**20**
	Female	4	4	4	4	4	**20**
Week 9	**Male**	4	4	4	4	4	**20**
	Female	4	4	4	4	4	**20**
Total sampled	**Male**	**17**	**17**	**17**	**17**	**17**	**85**
	Female	**17**	**17**	**17**	**17**	**17**	**85**

CBM: Cocoa bean meal

Similar random sampling of fish in each treatment for histological studies of the gonads were further done on fortnightly (2 weekly) basis for 3 more times (total number of 5 sampling through a 9 weeks period). Body weight and body length measurements of the fish in the different treatments were taken on a weekly basis for the entire duration of the study. The experimental layout used in the study and the sampling pattern of the fish for histology studies are presented in Table 14 and 15 respectively.

3.6 THE EXPERIMENTAL DIETS

The composition of the experimental diets used in the study is presented in table 16. The crude protein level used for the diet formulation was 35%. The diet fed to treatment 1 which served as the control group had no inclusion of cocoa bean meal. Diets of treatment 2, 3, 4, and 5 had cocoa bean meal (CBM) inclusion at the levels of 10, 20, 40, and 50% i.e. 10kg, 20kg, 40kg, and 50kg CBM in 100kg diet respectively.

Table 16: percentage composition of the experimental diet

Ingredients (kg)	T1 (Control)	T2	T3	T4	T5
Maize	26.88	22.39	17.64	8.13	3.38
Wheat offal	11.52	9.59	7.56	3.49	1.45
SBM	34.56	32.41	30.48	26.63	24.70
Fish meal	17.28	16.21	15.24	13.32	12.35
Blood meal	5.76	5.40	5.08	4.39	4.12
Cocoa bean meal	0	10	20	40	50
Bone meal	3	3	3	3	3
Salt	0.5	0.5	0.5	0.5	0.5
Methionine	0.25	0.25	0.25	0.25	0.25
Vit-min premix	0.25	0.25	0.25	0.25	0.25
Palm oil					
Total	**100**	**100**	**100**	**100**	**100**
Calculated composition					
Crude protein (%)	35	35	35	35	35
Crude fiber (%)	4.07	3.89	3.69	3.34	2.87
Metabolisable energy (Mcal/Kg)	2.51	2.46	2.41	2.31	2.03

***Each kg of vit-premix contains: vit.A, 10,000IU; vit. D3, 2,500IU; vit. E, 20mg; vit. K3, 3mg; thiamine, 2mg; riboflavin, 5mg; pyridoxine, 5mg; vit. B12, 0.015mg; Nicotinicacid, 40mg; Pantothenicacid, 12mg; folicacid, 0.75mg; biotin, 0.05mg; Vit. C, 100mg; manganese, 70ppm; zinc, 60ppm; iron, 60ppm; iodine, 1ppm; copper, 8ppm; selenium, 0.25ppm; cobalt, 0.15ppm**

3.7 DATA COLLECTION

During the course of the study various categories of data were collected. They include:

I. GROSS MORPHOLOGY

As earlier stated, the measurements of body length and body weight of fish were carried out at the onset of the experiment (before the commencement of the feeding trials). The wet body weight of the fish were measured using an electronic weighing scale (Atom A- 110C) to the nearest 0.01g. Both the total length (from the tip of snout to tail end) and standard length (from the tip of the snout to the line or point where the anal fin began), were measured to the nearest 0.01cm using a calibrated metric rule while the fish were placed on a flat board. Also on

fortnightly basis as described earlier, fish were collected from different treatments. Collected fish were weighed and the weights recorded. The fish were sacrificed and dissected to remove the gonads (testes and ovaries). The harvested gonads were weighed using electronic weighing scale to the nearest 0.01g, quickly observed and studied for morphological characteristics before they were fixed in Bouinøs solution for further laboratory preparations and histological studies. The following morphological data were collected in the study:

- Standard length
- Total length
- Fish wet weight
- Weight of gonads
- Gonadosomatic index (GSI)
- Gross ovarian morphology
- Gross testicular morphology

The The gonadosomatic index (GSI) were estimated according to the method described by Adebiyi *et al.* (2011) as:

$$GSI = \frac{weight\ of\ gonad\ (grams)\ \times 100}{weight\ of\ fish\ (grams)}$$

II. HAEMATOLOGY

Blood collection

Blood was collected from the fish on the 63rd day of the study. Two fish were sacrificed from each treatment group. About 0.5 millilitre of blood was drawn from the heart and the liver using a sterile disposable 2ml syringe. The collected samples were poured into an (ethylene diamine tetra-acetic acid) EDTA bottle and taken to the laboratory for analysis.

Methods of Analysis for Haematogical Parameters

(1) Hemoglobin (Hb %)

0.02ml of well mixed whole blood was diluted into four mls of drabkins solution. This is measured spectrophotometrically at 546 nanometer wavelength. After measuring the value of Hb was red from the calibration curve (calorimetrically) according to Van Kampen and Zillestra (1983).

(2) Pack Cell Volume (PCV)

Method: the PCV was determined by the microheamatocrit method (Coles, 1980). A micro capillary tube was nearly filled with the blood sample and sealed at one end. It was centrifuged at 10,000 revolutions per minute for 5 minutes using a microheamatocrit centrifuge. After centrifugation, the PCV was read using a microheamatocrit reader.

(3) WBC (mm^3) total white blood cell count:

Method: 0.02ml of blood is diluted with 0.38ml of diluting fluid. The total blood count is counted using improved neubauer counting chamber.

(4) RBCs – Red blood cells count:

Method: All counting methods are based on the dilution of capillary blood or well mixed, correctly anti-coagulated venous blood with counting fluids in a special counting pipette. The individual cells are counted in a counting chamber (hemocytometer). Alternatively, electronic counters such as the coulter counter may be used. The coulter counter counts the individual red blood cells of a measured volume of a diluted blood as they pass through a minute orifice guarded by an electric current flowing between platinum electrodes. Electronic counters work with a reproducibility of under $\pm$ 2%. Since the electronic counter enumerates red and white blood cells alike, an error will be introduced if there is a leukocytosis of over 30.000 cells/mm^3. In this case, the result may be corrected. With certain adjustments, the coulter counter can be also used to count platelets with a standard error of $\pm$ 2.85%. The cell counts are red directly from the counter.

Also at the end of the experiment, blood samples were collected from the fish for laboratory analysis.

- Packed cell volume
- Haemoglobin
- Red blood cell
- White blood cell
- Neutrophils
- Lymphocyte
- Monocyte
- Eosinophil
- Basophil

III. SERUM LIPID PROFILE

Blood samples were obtained from 10 samples (2 samples from each treatment) and the samples were allowed to clot at room temperature before being centrifuged to separate the serum and frozen prior to analysis.

Serum Total Cholesterol (TC): Serum TC concentration was measured by the end point colorimetric method (Allain et al., 1974) with the use of T60 spectrophotometer and test kits obtainable from Randox Laboratories Limited. In this method, the cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine was formed from the reaction between hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.

Serum High density lipoprotein (HDL): Serum HDL-Cholesterol was measured with a T60 spectrophotometer with test kits obtainable from Randox Laboratories Limited. In this method, low density lipoprotein and chylomicron fractions are precipitated quantitatively by addition of phosphotungstic acid in the presence of Mg^{2+} ions. After centrifugation, the cholesterol concentration in the HDL fraction which remained in the supernatant was then determined (Friedewald et al., 1972).

Serum Low density lipoproteins (LDL): Serum LDL-Cholesterol concentration was measured with a T60 spectrophotometer with test kits obtainable from Randox Laboratories Limited. In this method, low density lipoproteins are precipitated by heparin at their iso-electric point (pH=5.04). After centrifugation, the HDLCholesterol and VLDL remained in the supernatant. The cholesterol concentration in the LDL fraction was determined by enzymatic method (Wicland and Seidel, 1983).

Serum Triacyl glycerol: Serum Triacyl glycerol concentration was measured with a T60 spectrophotometer with test kits obtainable from Randox Laboratories Limited. In this method, the triglycerides are determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide, 4-amino phenazone and 4-chlorophenol under the catalytic influence of peroxidase (Tiez, 1990).

The serum lipid profile indices are:

- High density lipoprotein
- Low density lipoprotein
- Total cholesterol
- Triacylglycerides

IV. HISTOMORPHOLOGICAL STUDIES

This was done at the histology laboratory of the Faculty of Veterinary Medicine, University of Nigeria Nsukka, using the procedures described be Onuoha (2010). Fish gonads (testes and ovary) were collected and fixed for histological studies on fortnightly basis for two months. Also, tissue sections from the ovary and testes were prepared for histomorphology as stated previously. The tissues were dehydrated in graded concentrations of ethanol (serially from 70%, 80%, 90% and 100%) to remove the water contained in the tissues, cleared in xylene and embedded in paraffin wax. The blocks of tissue-embedded-paraffin-wax were produced and 5µm thick sections were cut from the blocks (using a rotary microtome), mounted on glass slides and stained with Hematoxylin and Eosin (H&E). The stained slides were viewed using MOTIC® binocular light microscope. Histomorphological observations and study were made for the oocyte and seminiferous tubules for the female and male fish respectively.

The procedures for fixing and embedding of tissue samples are summarized as follows:

- **Fixing**

The tissue samples were fixed in 70% ethanol for 90minutes and subsequently in 80% ethanol, 90% ethanol, and absolute ethanol for 90minutes at each stage to dehydrate the tissues.

- **Clearing**

The tissue samples were then cleared of alcohol using graded levels of chloroform (chloroform I and chloroform II) for 90minutes each.

- **Embedding**

The samples were transferred to paraffin wax I and II and left for 90minutes each to cause paraffin infiltration of the tissues. Then they were transferred into fresh molten wax in square wooden boxes with metal base. The block boxes were placed in trays with the face in contact with ice for cooling.

- **Histological section**

Sections of the tissues were made from the embedded tissues at 5µm thickness using a microtome and placed on consecutive slides. The sectioning of each sample was done using

standard histological procedures as described by Luna (1992), Cek *et al*. (2001) and Cek (2006)

- **Staining**

The sections were stained with Meyerøs Haematoxylin and Eosin (H & E) according to the modified procedures of Bancroft and Stevens (1991), and studied for the histologic parameters listed above.

V. HISTOMORPHOMETRICAL STUDIES

The gonadal tissues (testes and ovaries) were prepared using the same procedure as described for the Histomorphology. Histomorphometric evaluations were made using an ocular micrometer previously calibrated with a stage micrometer at magnifications of X100 and X400. Photomicrographs of the tissue sections were taken using 6 megapixels MOTIC® microscope camera at magnifications X100 and X400. Hematoxylin & Eosin (H&E) stained ovarian tissue sections from all the groups and their replicates were used for the evaluations. The different stages of oocyte development were identified in each slide as described by Saka and Adeyemo (2015) for African catfish (*Clarias gariepinus*). A minimum of five (5) round or nearly round oocytes for each of the developmental stages identified in each slide were randomly selected and the diameters obtained using an ocular micrometer calibrated at X100 magnification. The obtained figures were converted to millimeters (mm). Also, the stages of testicular development were identified as described by Saka *et al*. (2015). The tubular diameter and epithelial height were also measured for the testes and the measurements converted to millimeter. The data collected at this stage include:

- Oocyte diameter and distribution
- Tubular diameter and height

VI. GONADAL DEVELOPMENT

Gonadal development is determined using motic image (2.0) micro-photograph software connected to a computer and high power microscope with images being captured with the aid of zoom lens linked to a computer using the image capture software. The ovaries of the fish were evaluated according to the stages of gametogenesis. The ovary were grouped into four stages

depending on the pre-dominant stage and cell type in the tissue: Stage I, Stage II, Stage III, and Stage IV. Spermatozoa classification was based on the histological criteria adapted from Grier (1981). Oocytes were classified by developmental stages adapted from Bromage and Cumaranatunga (1987).

3.8 EXPERIMENTAL DESIGN

The Experiment was carried out in a Randomized complete block Design (RCBD). The mathematical model adopted was:

$$\gamma_{ijk} = \mu + T_i + \beta_j + (\tau\beta)_{ij} + \sum_{ijk}$$

Where γ_{ijk} = any observation or measurement taken

μ = population mean

T_i = Treatment effect

β_j = Effect of the jth sex

$\sum_{ijk}$ = Experimental error

3.9 STATISTICAL ANALYSIS

Data generated was subjected to analysis of variance (ANOVA) using statistical package (SPSS. version 20) and significantly different means were separated according to the procedures of Duncanøs New Multiple Range Test (Duncan, 1955).

CHAPTER FOUR

RESULT AND DISCUSSION

4.1: GROWTH PERFORMANCE

The result of the growth performance of *C. gariepinus* catfish fed diets containing varying levels of cocoa bean meal is presented in Table 17.

Table 18: Effect of dietary inclusion of cocoa bean meal on growth performance of *C. gariepinus* catfish

Indices	T1 (Control)	T2 (10% CBM)	T3 (20% CBM)	T4 (40% CBM)	T5 (50% CBM)	SEM
Av. In. BW (g)	23.57	23.80	22.90	23.99	22.19	0.53^{NS}
Av. Fin. BW (g)	27.16^{b}	32.27^{a}	27.93^{ab}	29.54^{ab}	23.17^{c}	1.06*
Av. BW. Gain (g)	3.59^{b}	8.44^{a}	5.03^{ab}	5.55^{ab}	0.98^{c}	0.95*
Av.Ini. SL (cm)	14.16	14.02	13.80	13.73	13.77	0.13^{NS}
Av. Fin. SL (cm)	15.23	15.89	14.99	15.27	14.84	0.23^{NS}
Av.SL gain (cm)	1.07	1.87	1.08	1.54	1.06	0.22^{NS}
Av.Ini. TL (cm)	15.95	15.81	15.78	15.73	15.72	0.12^{NS}
Av. Fin. TL (cm)	17.26	18.00	16.99	17.35	16.84	0.25^{NS}
Av.TL gain (cm)	1.32	2.20	1.21	1.61	1.13	0.25^{NS}

[abc] Row means with different superscripts are significantly (P<0.05) different; NS: Non significant; Av. In BW: Average Initial body weight; Av. Fin. BW: Average Final body weight; Av. BW. Gain: Average body weight gain; Av. In. SL: Average initial standard length; Av. Fin. SL: Average final standard length; Av. SL gain: Average standard length gain; Av. In. TL: Average initial total length; Av. Fin. TL: Average final total length; Av. TL gain: Average total length gain

From the result, Av. Fin. BW and Av. BW. Gain were significantly (P<0.05) different among the treatment groups. There were no significant (P×0.05) difference among the treatments in Av. Fin. SL, Av. SL gain, Av. Fin. TL, and Av. TL gain. The fish in T2 (10% CBM) had the highest significant values for Av. Final BW and Av. B.W. Gain (32.27 and 8.44g respectively). The control (T1) and T5 (50% CBM) had significantly lower values than the T2 (10% CBM) in their final body weight as well as weight gain. The mean values of T3 (27.93 and 5.03g respectively) and T4 (29.54 and 5.55g respectively) for Av. Final Wt. and Av. Wt. gain did not differ significantly (P×0.05) from each other and from T2 and T1. Mean body weight of the fish ranged from 22.19 to 32.27 which is similar to the values reported by Ozovehe (2013) and Jimoh *et al*. (2014) for juvenile *C. gariepinus* catfish. The average body weight gain were similar to those reported by Adebowale and Olubamiwa (2008) (1.73 ó 4.11g) when up to 25% cocoa husk endocarp was fed to *C. gariepinus* catfish for 12 weeks. Average final body weight and average weight gain significantly (P<0.05) increased in T2 (10% CBM) and decreased as the levels of

CBM increased in the diet. The higher values obtained for T2 when compared to the control group could be attributed to the antioxidant properties of cocoa. Polyphenols are known to act as proton donor-scavenging radicals (Rice-Evans *et al*., 1997), inhibition of enzymes that increase oxidative stress, chelates metals, bind carbohydrates, and proteins (Helm *et al*., 2002). The higher mean final weight and weight gain observed for the fish in T2 may be attributed to the flavonoids and the anti-oxidant properties cocoa. On the other hand, the significantly ($P<0.05$) lowered average final body weight and average body weight gain values observed in T5 (50% CBM) may be attributed to theobromine. Its level in cocoa bean cake ranges from 20 to 23g/kg (Laconi, 2009). Theobromine has been reported as an anti-nutritional factor which is capable of suppressing appetite, reducing feed intake and hence reducing growth performance (Muhammed *et al*., 2000; Alexander *et al*., 2008). This may be the reason why theobromine has been considered useful for weight loss and is supplemented to herbal tea preparations (Khazan *et al*., 2014). Similarly Ayinde *et al*. (2010) reported that inclusion of cocoa bean shell above the level of 100g/kg in the diet of rabbits adversely affected body weight. In broiler chicken, reduced growth was observed when cocoa bean shell incorporated in the diet exceeded 10% (Teguia *et al*., 2004).

4.2 OVARIAN DEVELOPMENT

4.2.1 Gross morphology and description of ovarian stages

The stages of ovarian development were characterized according to the reports of Owiti and Dadzie (1989) and Saka and Adeyemo (2015). The following 4 developmental stages (stages 1, 2, 3, and 4) of the ovary based on their gross morphology were identified during the study. No stage 5 ovary was observed during the course of the study.

Stage I: The ovary at this stage was found attached to the dorsal-lateral region of the peritoneum. They were observed as thin, tube-like, hollow structure which is translucent and creamy white in colour. In some of the fish, it was found covered by some white body adipose tissue. It was difficult to differentiate it from the testes, but with a closer observation or under a magnifying lens, it was different from the testes due to its smooth edges unlike those of the testes which were serrated. This was seen in some of the sampled fish by the 15th week of age when the study commenced.

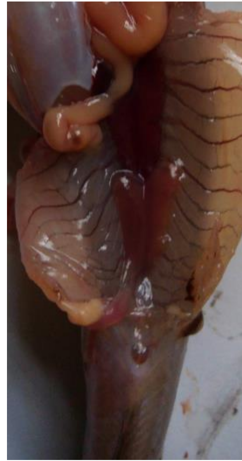


Plate 5: Gross morphology of the stage 1 ovary

Stage II: ovaries in this stage are granular and reddish brown in colour. They continuously increase in size embedded to the peritoneal cavity. At the start of this stage the ovaries are smooth and clear but towards the end they appear completely filled with oocytes of different sizes which are clearly visible to the unaided eye. These oocytes appear as yellowish-green or brownish yellow granules in the ovary. Owiti and Dadzie (1989) classified this later stage 2 as stage 3 ovary. Saka and Adeyemo (2015) however maintained it was a later stage of stage II.

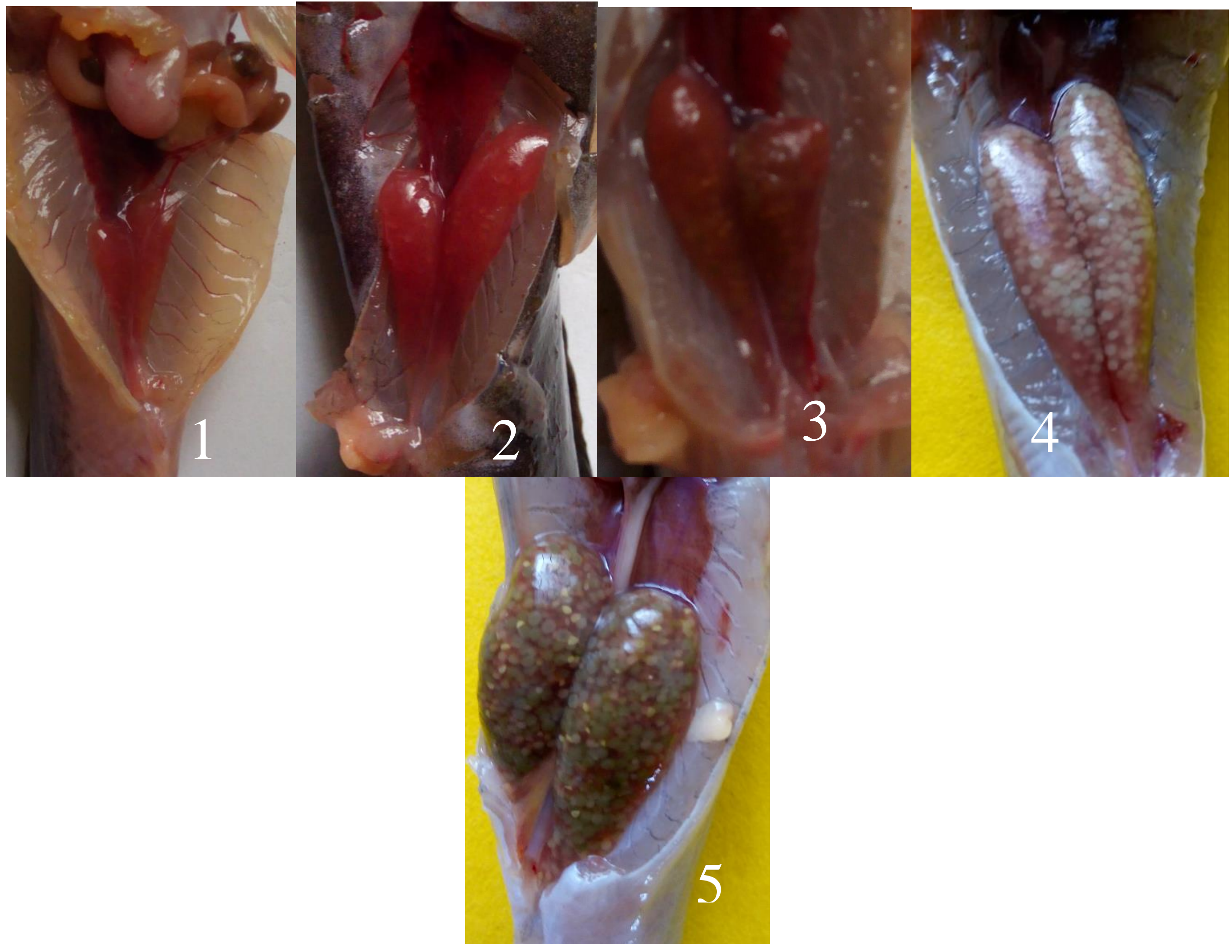


Plate 6: Gross morphology of the stage II ovary. 1, 2, and 3 represents the early stages of stage 2, whereas 4 and 5 are the late/ advanced stage II.

Stage III (Maturation stage): The ovary at this stage occupied a greater portion of the peritoneum. They are ovaries that are large and usually matt green at the beginning of this stage but becomes clear green at the peak of the stage. There is pronounced vascularization around the ovary and the oocytes are still seen within the lamellae while the lumen of the ovary is empty. This ovary at this stage is characterized by fully vitellogenic oocytes (i.e. the yolk vesicle and the yolk granule oocytes) and are seen in females close to spawning period.

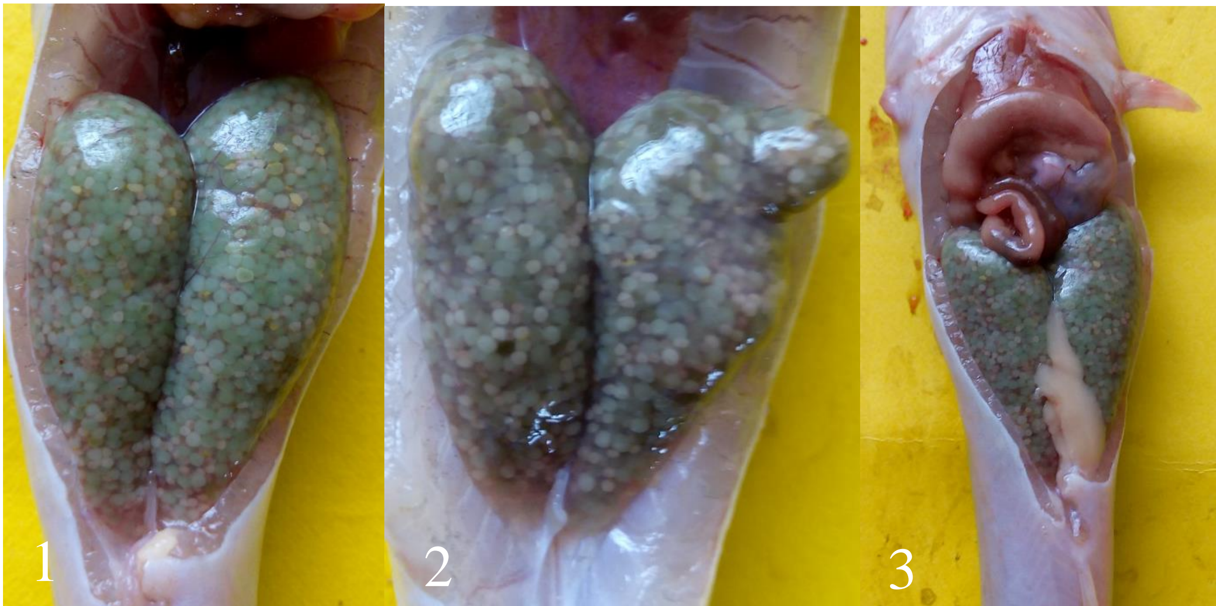


Plate 7: Gross morphology of stage III ovary showing the matt green ovarian structure which is occupying ¾ of the abdominal cavity

Stage IV (Ripe/ mature): this represents the stage of maximal ovarian development. The ovaries in this stage are transparent clear green and are nearly identical to those of stage 3. They are also seen as opaque and brown green containing eggs visible to the naked eye. The oocytes are partially ovulated and can be expelled with a gentle pressure on the fish flanks. During this stage, yolk granule oocytes predominate.

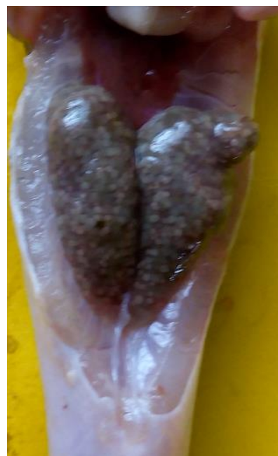


Plate 8 : The pictograph showing the Gross morphology of the stage IV ovary

4.2.2 Effect of varying dietary levels of cocoa bean meal on the ovarian development of *C. gariepinus* catfish.

The result of the effect of varying dietary levels of cocoa bean on ovarian development of *C. gariepinus* catfish is presented in Table 18.

Table 19: Ovarian development of *C. gariepinus* catfish fed varying dietary levels of cocoa bean meal

Weeks	indices	T1 (Control)	T2 (10% CBM)	T3 (20% CBM)	T4 (40% CBM)	T5 (50% CBM)	SEM
3	**B. W (g)**	17.53	18.96	17.17	16.72	18.86	1.32^{NS}
	O. W (g)	0.121^{ab}	0.178^{a}	0.142^{a}	0.112^{ab}	0.045^{b}	0.013*
	GSI	0.759^{a}	0.887^{a}	0.806^{a}	0.765^{a}	0.203^{b}	0.077*
5	**B. W (g)**	19.13	19.52	19.14	16.95	20.11	1.312^{NS}
	O. W (g)	0.182	0.178	0.184	0.196	0.111	0.014^{NS}
	GSI	0.942	0.914	0.819	0.806	0.534	0.062^{NS}
7	**B. W (g)**	21.62	25.84	23.08	24.33	20.46	1.695^{NS}
	O. W (g)	0.204	0.223	0.174	0.220	0.12	0.022^{NS}
	GSI	1.079	0.927	0.927	1.16	0.639	0.094^{NS}
9	**B. W (g)**	33.24^{ab}	37.73^{a}	34.47^{ab}	28.24^{b}	29.33^{b}	1.652*
	O. W (g)	0.358^{b}	2.356^{a}	0.579^{b}	1.269^{ab}	0.359^{b}	0.175*
	GSI	1.124^{b}	6.22^{a}	1.646^{b}	3.951^{ab}	1.207^{b}	0.439*

[abc] Row means with different superscripts are significantly (P<0.05) different B. W.: Body weight; O.W.: Ovary weight; GSI: Gonadosomatic index; NS: Non-significant.

Form the study, it was observed that after the first three weeks of the study, gonad weights (GW) and gonadosomatic index (GSI) of the female fish differed significantly (P<0.05) among the treatment groups. Whereas body weight was not significantly (P×0.05) different among the treatment groups. T2 and T3 had the highest values for G.W. (0.178 and 0.142g) respectively. On the other hand, T5 had the least significant value for gonad weight (0.045). Although higher values than was obtained from T5 were observed for T4 and the control groups, they were not significantly different from those of T5, T2, and T3. Also, T2 had the highest GSI values (0.887) which was not significantly different from T3, T4, and control (0.806, 0.765, and 0.759 respectively). T5 had the least GSI value of 0.203 which was significantly lower than those of the other treatment groups. At weeks 5 and 7 of the study, there was no significant difference

among the treatment groups. At week 9 however, body weight, gonad weight and GSI values were significantly ($P<0.05$) different among the treatment group. In the body weight values, T2 had the highest value (37.73g) which was significantly different from T4 and T5 (28.24 and 29.33g) which had the least body weight values. T3 and the control with the respective body weight values of 34.47 and 33.24g did not differ significantly from T2, T4, and T5. Also, T2 had the highest G.W and GSI values (2.356g and 6.22 respectively). The least G.W. and GSI values were observed in T3 (0.579g and 1.646 respectively), T5 (0.359g and 1.207 respectively) and the control (0.358g and 1.124) groups. T4 had G.W. and GSI (1.269g and 3.951) values which did not differ from those of the other groups. The results of this study show that after a short time of feeding the experimental diet of cocoa bean meal, it had effects on gonad weight and GSI. And at up to 40% dietary inclusion, these indices were found not to be adversely affected. However, at 50% inclusion in the diet, the ovarian development as measured by the ovarian weight and GSI were adversely affected. As the feeding continued from the 3rd to the 7th week, the ovarian development continued at the similar rate in all the treatment groups. Hence, there were no significant ($P\times0.05$) differences among the treatments. However, as the feeding trial continued, at the 9th week, T2 was seen to be more developed than those of the other treatments as seen by their highest body weight (37.73g), gonad weight (2.356g), and GSI (6.22) values. The least values were obtained for the control, T3, and T5. The T4 however, was not seen to differ significantly from T2 and the other groups. At this period (24 weeks of age), the G.W were found to range from 0.358g in the control group to 2.356g in the T2 group (10% CBM). The GSI also range from 1.124 in the control group to 6.22 in T2. Thus, cocoa bean meal inclusion in the diet of *Clarias gariepinus* catfish, although did not affect gonad development of the female fish at short feeding duration, continued feeding up to 9 weeks was shown to improve the body and gonadal weight of the fish. Results of the GSI values were similar to those reported by Oyelese (2004) of 7.66 when female *Clarias gariepinus* catfish were fed diets containing 35% crude protein.

Results also show that cocoa bean meal inclusion in the diet of *Clarias gariepinus* catfish improved the ovarian growth and development as reflected in the gross morphology of the ovary in the week 3 (plate 9) and week 9 of the experiments (plate 10). At week 3, the ovaries of T1 (control), T2 (10% CBM), T3 (20% CBM), and T4 (40% CBM) were similar to each other in the gross morphologic development. They appeared to be more developed than the ovaries of the fish in T5 (50% CBM). At week 9, the ovaries of the fish in T2 and T4 were observed to be

better developed (stage IV) when compared to other treatment groups. The ovary of the fish in the control group were seen to be in stage II of the ovarian development while those of T3 (20% CBM) and T5 (50% CBM) were observed to be in stage III of ovarian development. Similarly, Dada and Adeparusi (2012) reported enhanced reproductive performance (reflected by improved fecundity, ovary weight, egg size and GSI) when of *C. zambesicus* and *S. indicum* seed powder were included in the diet of *C. gariepinus* at levels of 200g/kg diet. Also, Adeparusi *et al*. (2010) used *Kigelia africana* as a fertility enhancer in *C. gariepinus*. Dada (2012) also reported improved reproductive performance of catfish *C. gariepinus* broodstocks fed on diets supplemented by medicinal plants than those fed with the control diet. Furthermore, similar results were reported for using medicinal plants as fertility-promoting agents for catfish *C. gariepinus* (Dada and Ajilore, 2009; Dada *et al*., 2010; Dada and Ogunduyile, 2011; Dada, 2012). The better ovarian development of the *C. gariepinus* female catfish obtained in this study at lower levels of 10 and 40% CBM (T2 and T4 respectively) could be as a results of the presence of flavonoids in cocoa powder. Reactive oxygen species (ROS) and antioxidant enzyme systems are important components of many reproductive processes, including ovarian follicular development, ovulation, and fertilization (Al-Gubory *et al*., 2010). Oxidative stress reflects an imbalance between the generation of ROS/free radicals (example superoxide radical, hydroxyl radical and hydrogen peroxide) and antioxidant defences [example copper-zinc superoxide dismutase (SOD) and manganese SOD] which can result in damage to DNA, proteins and lipids (Sugino *et al*., 2007). These adversely affects oocyte development and ovarian growth. According to Ly *et al*. (2014), sufficient maternal antioxidant status may help prevent and/or manage adverse mechanisms intimately related to poor reproductive performances and that are also associated with poor dietary habits and oxidative stress. Also, Dada and Adeparusi (2012) reported that the presence of bioflavonoids (which are potent antioxidants) are capable of increasing the production of eostrogen, the key hormone involved in the production and maturation of eggs in the ovary. The pictograph of the gross morphology of the *C. gariepinus* ovary at weeks 3 and 9 of the study are shown in plates 9 and 10.

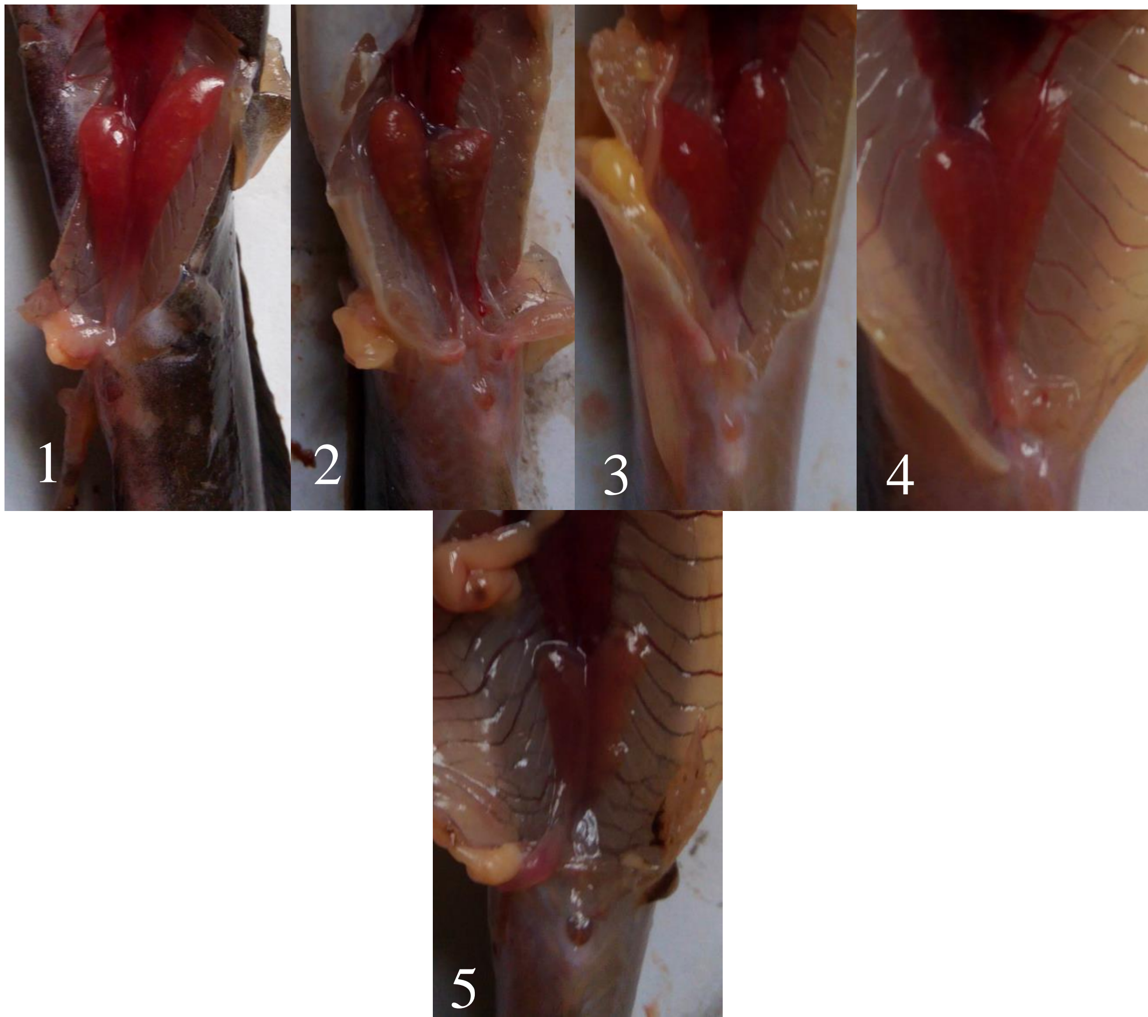


Plate 9: gross morphology of the *C. gariepinus* catfish ovary at week 3 of the experiment. 1, 2, 3, 4, and 5 represents treatments 1, 2, 3, 4, and 5 respectively. T1, T2, T3, and T4 which are in stage II are seen to be larger and more developed than the T5 which is seen to be in stage I of development.

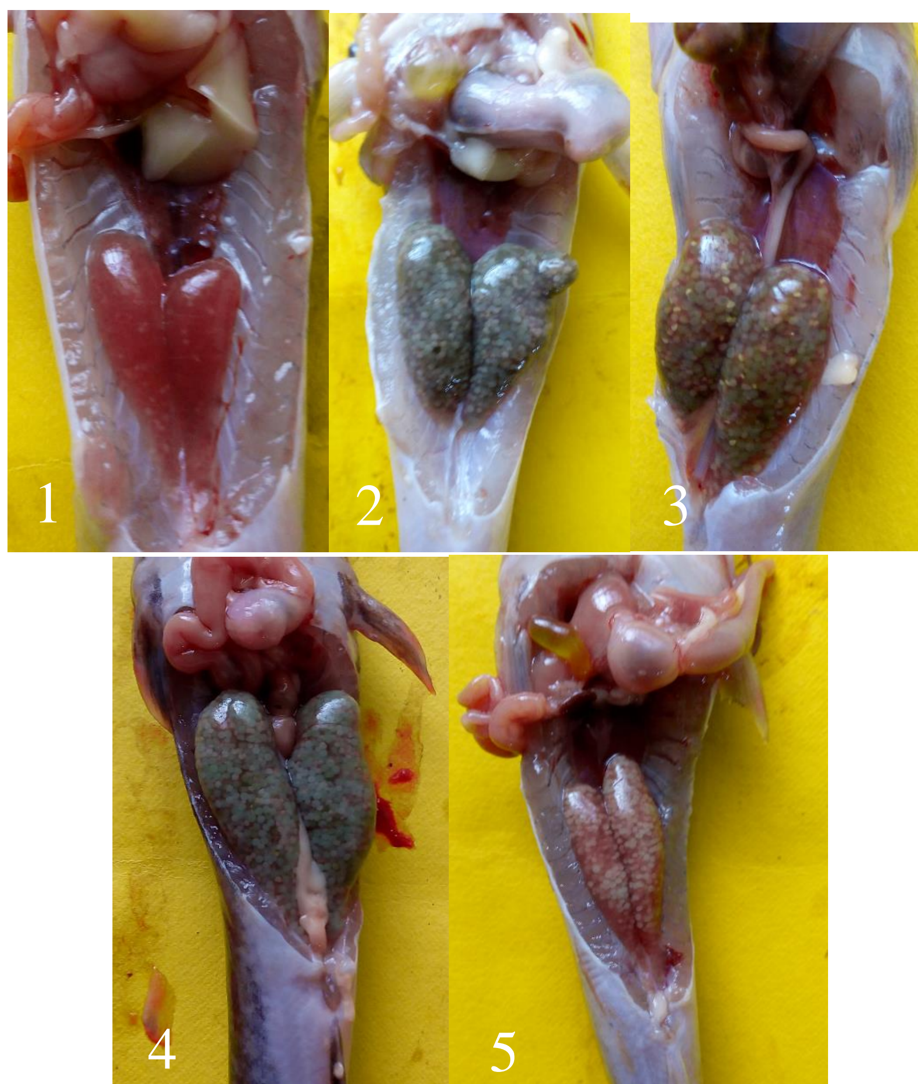


Plate 10: Gross morphology of the *C. gariepinus* ovary at week 9 of the experiment. 1, 2, 3, 4, and 5 represents treatments 1, 2, 3, 4, and 5 respectively. The ovaries are in stages of II (T1), early III (T5), late III (T3) and IV (T2 and T4) of development as classified by Saka and Adeyemo (2015)

4.2.3 OOCYTE DEVELOPMENT

Tissue samples from each group and their replicates were examined chronologically to determine their maturity stages. The ovarian maturity classes as previously described by Cek and Yilmaz (2007) and Saka and Adeyemo (2015) were observed in all the groups. The observed classes include; Primary growth phase (stages 1 and 2), Secondary growth phase (stages 3, 4 and 5), and Maturation/Hydration phase (stage 6). The identification and grouping of the observed oocytes into ovarian maturity classes were based on the appearance of the nuclei and nucleoli, and cytoplasmic (ooplasmic) characteristics. Based on the aforementioned, the following stages of oocytes were observed during the course of the study.

Stage 1 (Chromatin Nucleolar stage): This stage was characterized by a large nucleus in the central position, surrounded by thin deeply basophilic cytoplasm (ooplasm). The diameter of the observed oocytes in this group ranged from 0.12mm - 0.14mm.

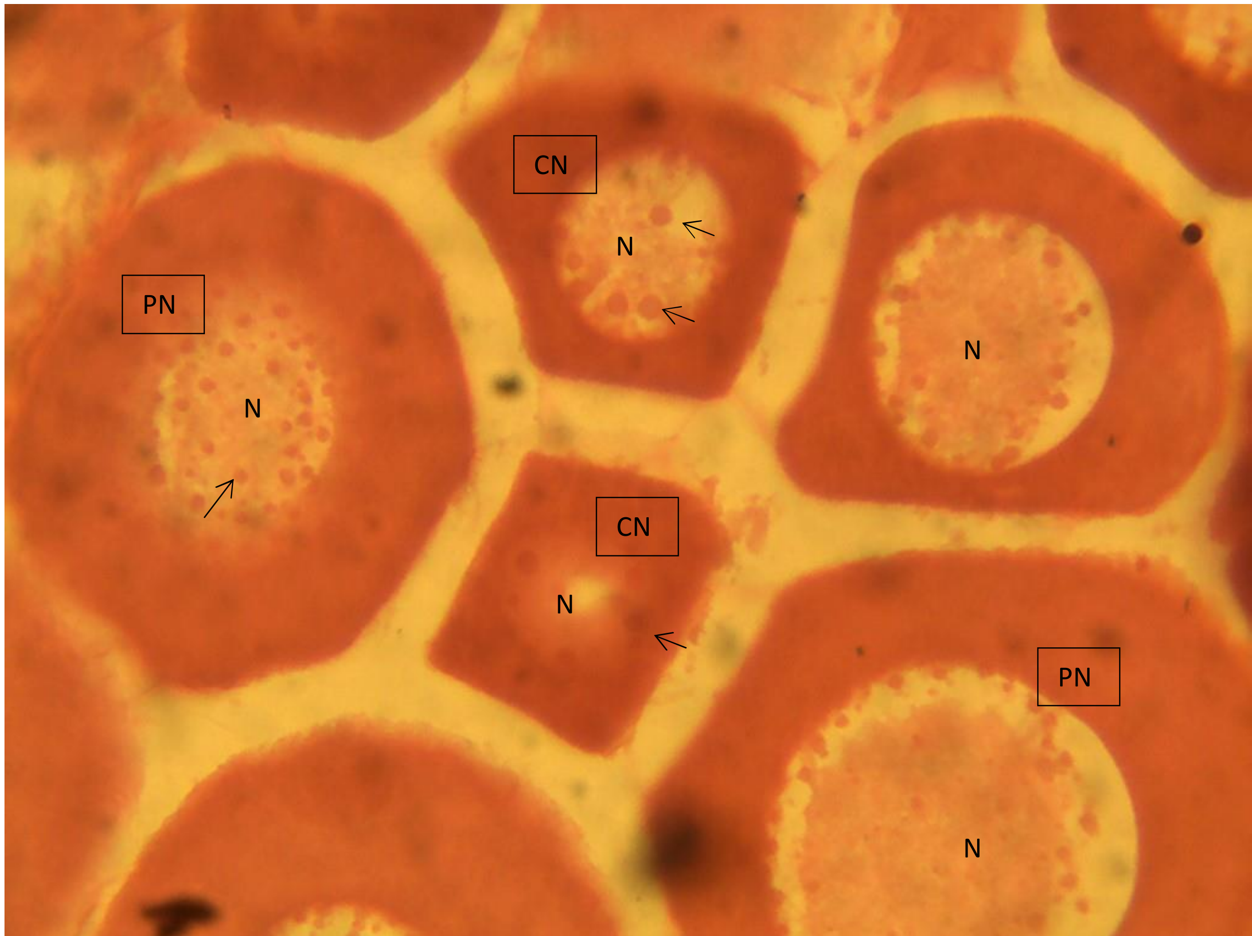


Plate 11: Photomicrograph of the ovary showing some of the stages of oocyte development. Present in the photomicrograph are oocytes in stages 1 (CN) and stage 2 (PN). Nucleus (N), Nucleolus (arrowed). H&Ex400

Stage 2 (Peri-nucleolar stage): Here, the nucleus increases in size and the nucleoli increases in number. Cytoplasm becomes lighter with multiple, dark, minute dot distributed all over the cytoplasm. The oocyte diameter ranged from 0.2mm ó0.40 mm.

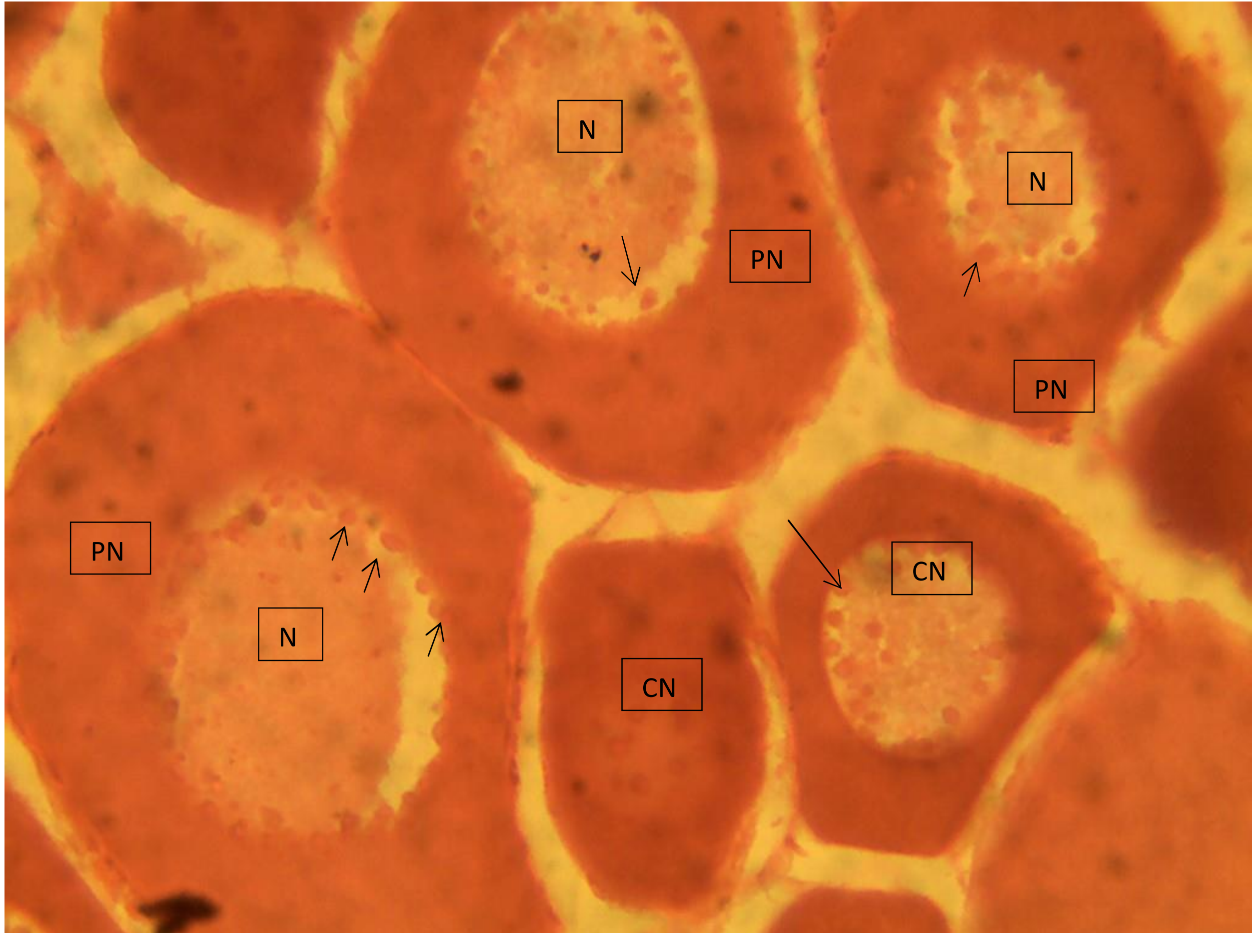


Plate 12: Photomicrograph of the ovary showing some of the stages of oocyte development. Present in the photomicrograph are oocytes in stages 1 (CN) and stage 2 (PN). Nucleus (N), Nucleolus (arrowed). H&Ex400

Stage 3 (Vessicle formation stage): The oocytes are generally bigger than the stage 2 oocytes and vesicles (observed as clear spaces) are present in the cytoplasm. These vesicles are called the cortical vesicles. They are multiple spherical clear vacuoles which usually appear as a single layer at the periphery of the ooplasm (cytoplasm). These vacuoles mark the initiation of the Secondary growth phase. The oocyte diameter at this stage ranges from 0.50mm ó 0.65 mm.

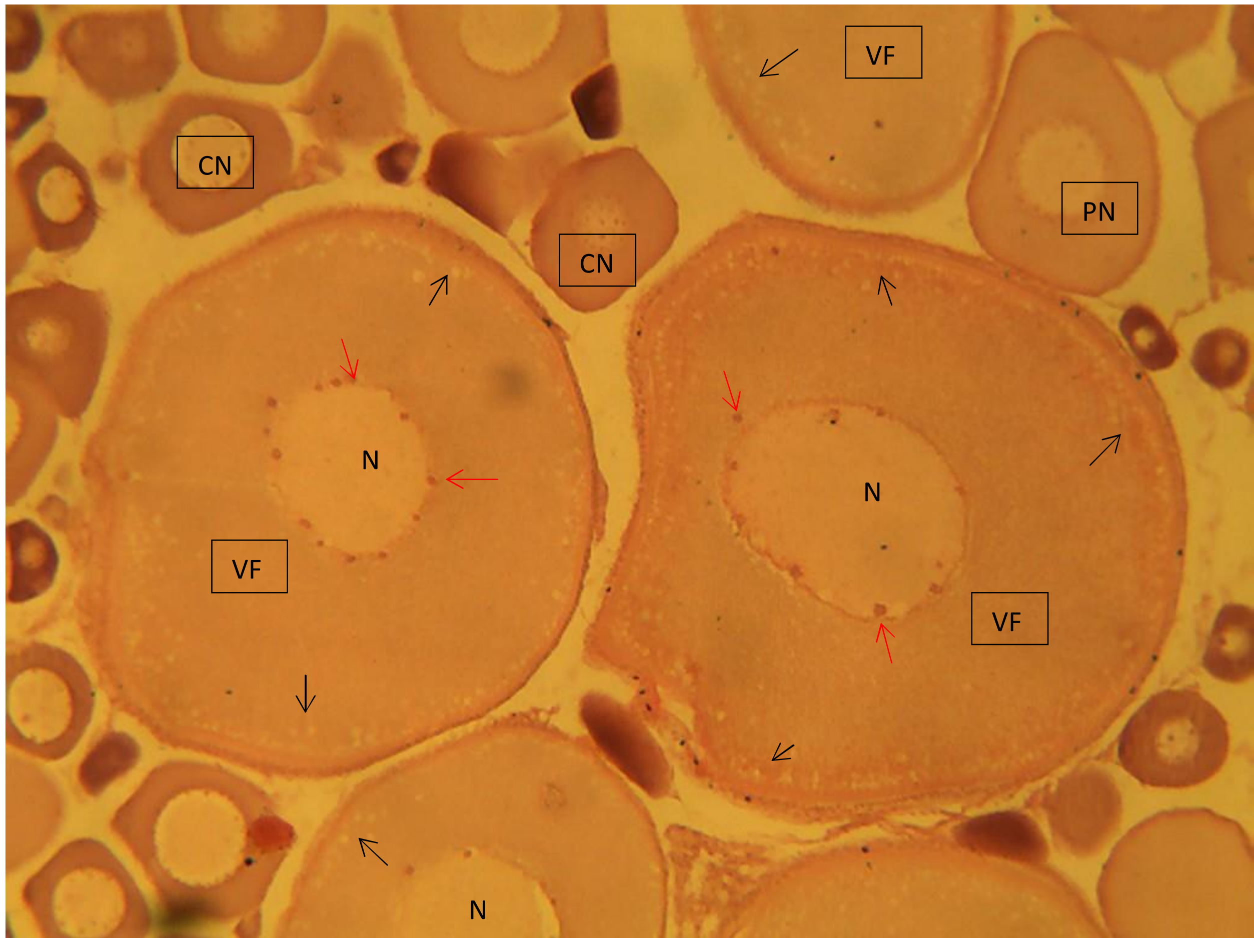


Plate 13: Photomicrograph of a section of the ovary, showing oocytes at varying developmental stages. Stage 1 (CN), stage 2 (PN) and stage 3 (VF) oocytes can be observed in the photomicrograph. Note the appearance of vesicles (black arrow) at the periphery of the cytoplasm of stage 3 oocytes. Also note the fewer nucleoli. Nucleus (N), Nucleoli (red arrow). H&Ex400.

Stage 4 (Exogenous yoke formation stage): Here, the oocytes are generally larger than those of the previous group. The nuclei are enlarged, consisting of many nucleoli. The Zona radiata is more conspicuous than what is observed in the stage 3 oocytes. The cortical vesicles are usually observed to be in multiple layers (usually 2 -3 layers) at the periphery of the cytoplasm (ooplasm). The oocyte diameter ranged from 0.77mm ó 0.97 mm.

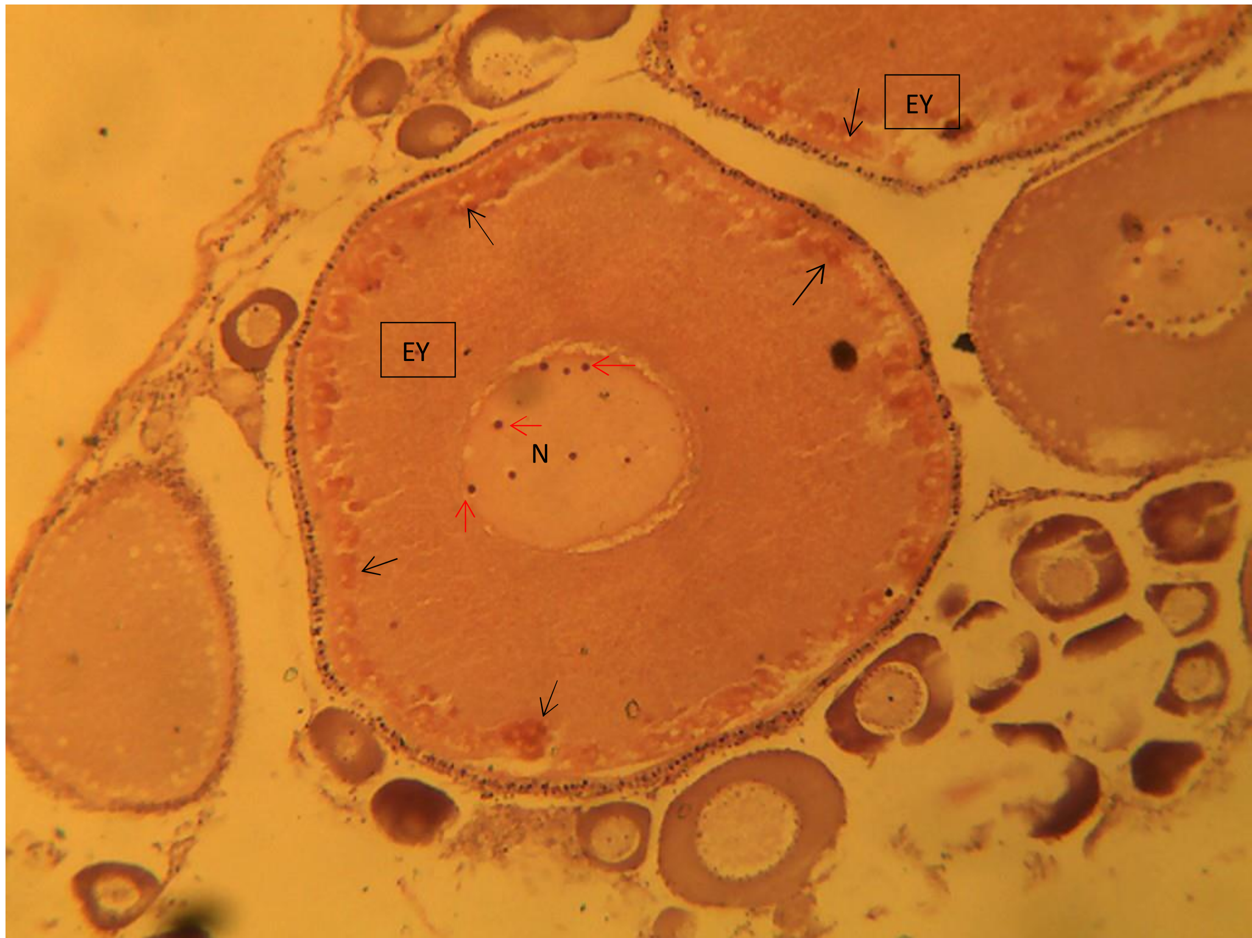


Plate 14: Photomicrograph of the ovary, showing oocytes in the 4th stage of development. In the stage 4 oocyte (EY), note the appearance of yolk globules which form a pinkish ring (black arrow) admixed with the peripherally arranged cytoplasmic vacuoles. Also notice the much fewer nucleoli in the stage 4 oocyte (red arrow). Nucleus (N). H&Ex400.

Stage 5: They are slightly larger than stage 4 oocytes. The nucleoli are much smaller in number as the nucleus becomes more irregular and smaller in size. Yolk granules are present in the cytoplasm, initially admixed with the layers of the cortical vesicles and later involving a wider part of the cytoplasm, causing the cytoplasm to appear granular under H&E. This causes the cytoplasm to stain more eosinophillic than observed in the initial stages. The oocyte diameter ranged from 1.07mm ó 1.12 mm.

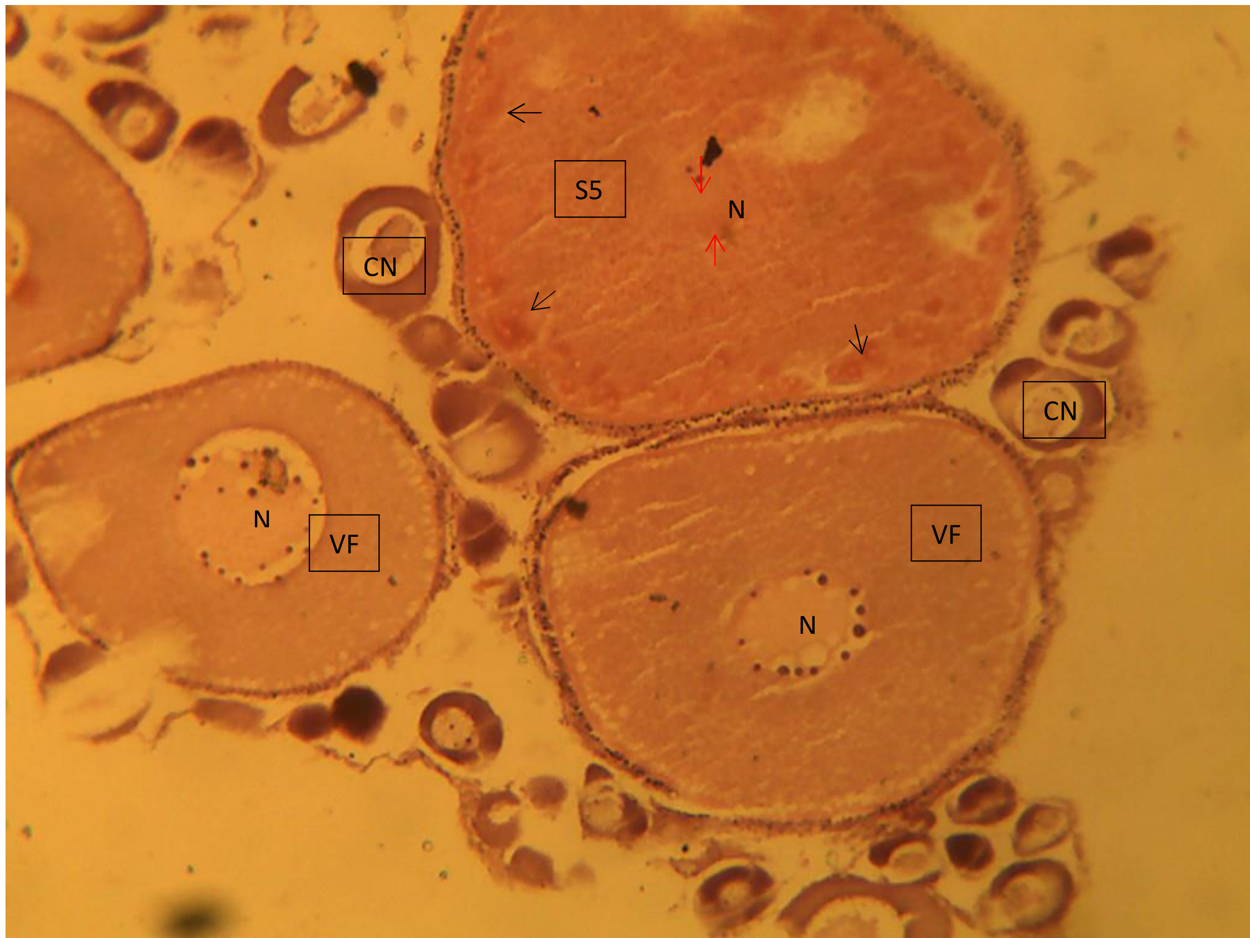


Plate 15: Photomicrograph showing oocyte in stage 5 (s5) of development flanked by oocytes of the primary growth phase. Note the granular nature of the cytoplasm due to the formation of yolk granules (black arrow). Also note the irregular inconspicuous nucleus (N) with few nucleoli (red arrow). Stage 3 oocyte (VF), Stage 1 oocyte (CN). H&Ex400

Stage 6: The oocytes were largest. The nuclei were fairly irregular, throwing projections into the cytoplasm. The nucleoli were few in number and inconspicuous. The cytoplasms were filled with yolk granules which cause the cytoplasm to appear more granular, staining more eosinophillic. The oocyte diameter ranged from 1.15mm -2.19mm.

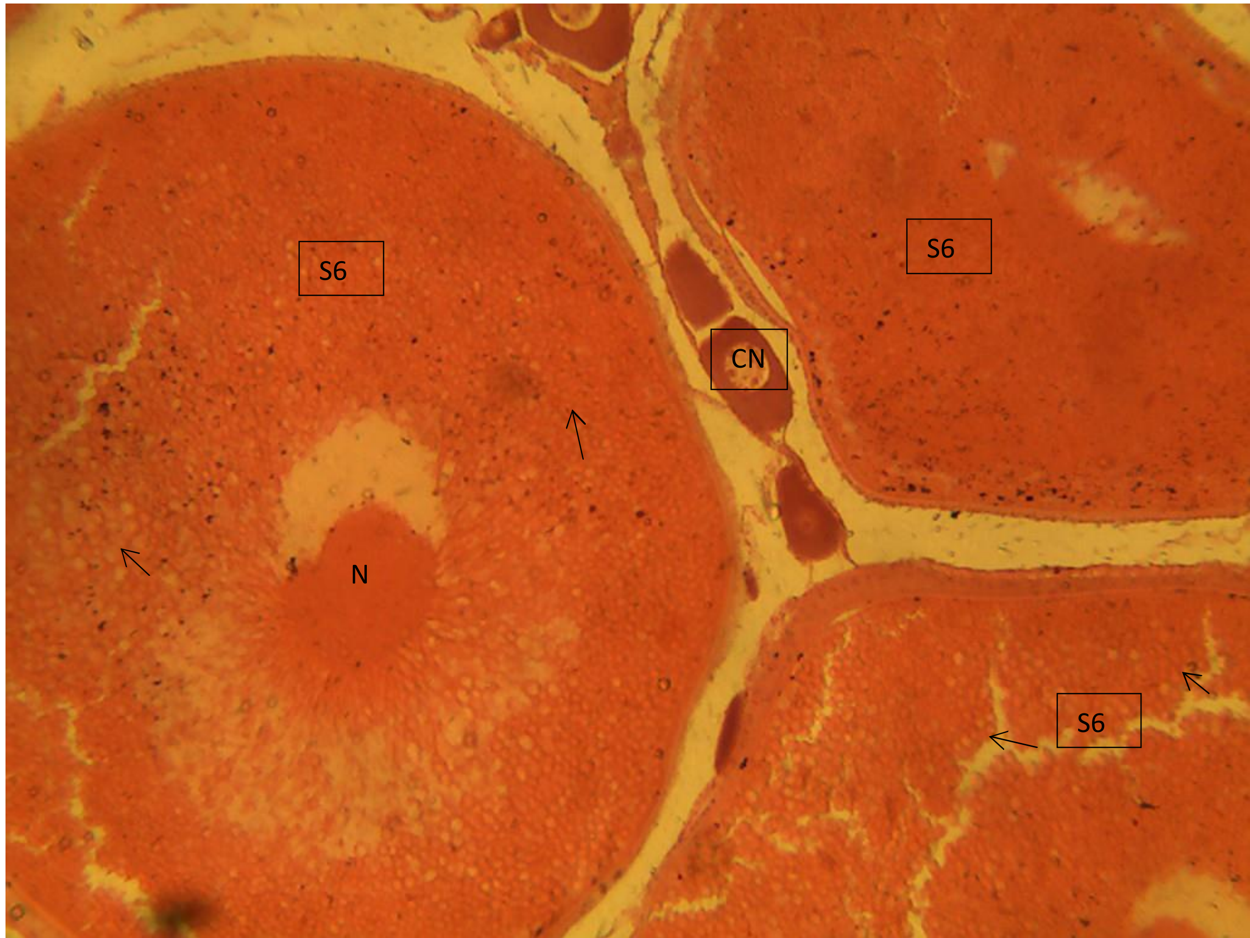


Plate 16: Photomicrograph of the ovary showing oocytes in stage 6 (S6). Note the granular nature of the cytoplasm due to the presence of yolk granules (arrow). Also note the irregular shape of the nucleus (N) and its projections into the cytoplasm. Stage 1 oocyte (CN). H&Ex400.

4.24 Effect of varrying dietary inclusions of cocoa bean meal (cbm) on oocyte distribution in *C. gariepinus*

Oocyte distribution for the different treatment groups at the 3rd, 5th, 7th and 9th week post treatment are summarized in the Table 19.

Table 20: Oocyte distribution for the different treatment groups at the 3rd, 5th, 7th, and 9th week post treatment.

	GROUP T1 (Control)	GROUP T2 (10% CBM)	GROUP T3 (20% CBM)	GROUP T4 (40% CBM)	GROUP T5 (50% CBM)
WEEK 3	Stages 1, 2, 3 and 4 oocytes were the predominant stages observed. A few Stage 5 oocytes were present	Stages 1, 2, 3 and 4 oocytes were the predominant stages observed. A few Stage 5 oocytes were present	Stages 1, 2, 3 and 4 oocytes were the predominant stages observed. A few Stage 5 oocytes were present	Stages 1, 2, 3 and 4 oocytes were the predominant stages observed. A few Stage 5 oocytes were present	Stages 1, 2, 3 and 4 oocytes were the predominant stages observed. A few Stage 5 oocytes were present
WEEK 5	Stages 1,2,3,4 and 5 were observed. Substantial numbers of oocytes in their secondary growth phase were present	All the oocyte developmental stages were observed. However, oocytes in the maturation phase were few in population	Stages 1,2,3,4 and 5 were observed. Substantial numbers of oocytes in their secondary growth phase were present	Stages 1,2,3,4 and 5 were observed. Substantial numbers of oocytes in their secondary growth phase were present	Stages 1,2,3,4 and 5 were observed. Substantial numbers of oocytes in their secondary growth phase were present
WEEK 7	Stages 1,2,3,4 and 5 were observed. Substantial numbers of oocytes in their SGP were present. Oocytes in their PGP appears to be declining	All the oocyte developmental stages were observed. Oocytes in the SGP and Oocytes in the maturation phase (MP) were predominant.	Stages 1,2,3,4 and 5 were observed. Substantial numbers of oocytes in their SGP were present. Oocytes in the Maturation phase were few in number.	Stages 1,2,3,4 and 5 were observed. Substantial numbers of oocytes in their SGP were present. Maturation phase were present but few.	Stages 1,2,3,4 and 5 were observed. Substantial numbers of oocytes in their SGP were present.
WEEK 9	All the developmental stages were observed. Oocytes in the Maturation phase are few. Oocytes in the SGP are predominant.	Only oocytes in the PGP and MP were observed. Oocytes in the Maturation phase (stage 6) are predominant. Oocytes in the SGP are sparse.	Only oocytes in the PGP and MP were observed. Oocytes in the Maturation phase (stage 6) are predominant.	Only oocytes in the PGP and MP were observed. Oocytes in the Maturation phase (stage 6) are predominant.	Only oocytes in the PGP and MP were observed. Oocytes in the Maturation phase (stage 6) are predominant.

PGP: primary growth phase; SGP: secondary growth phase; MP: maturation phase

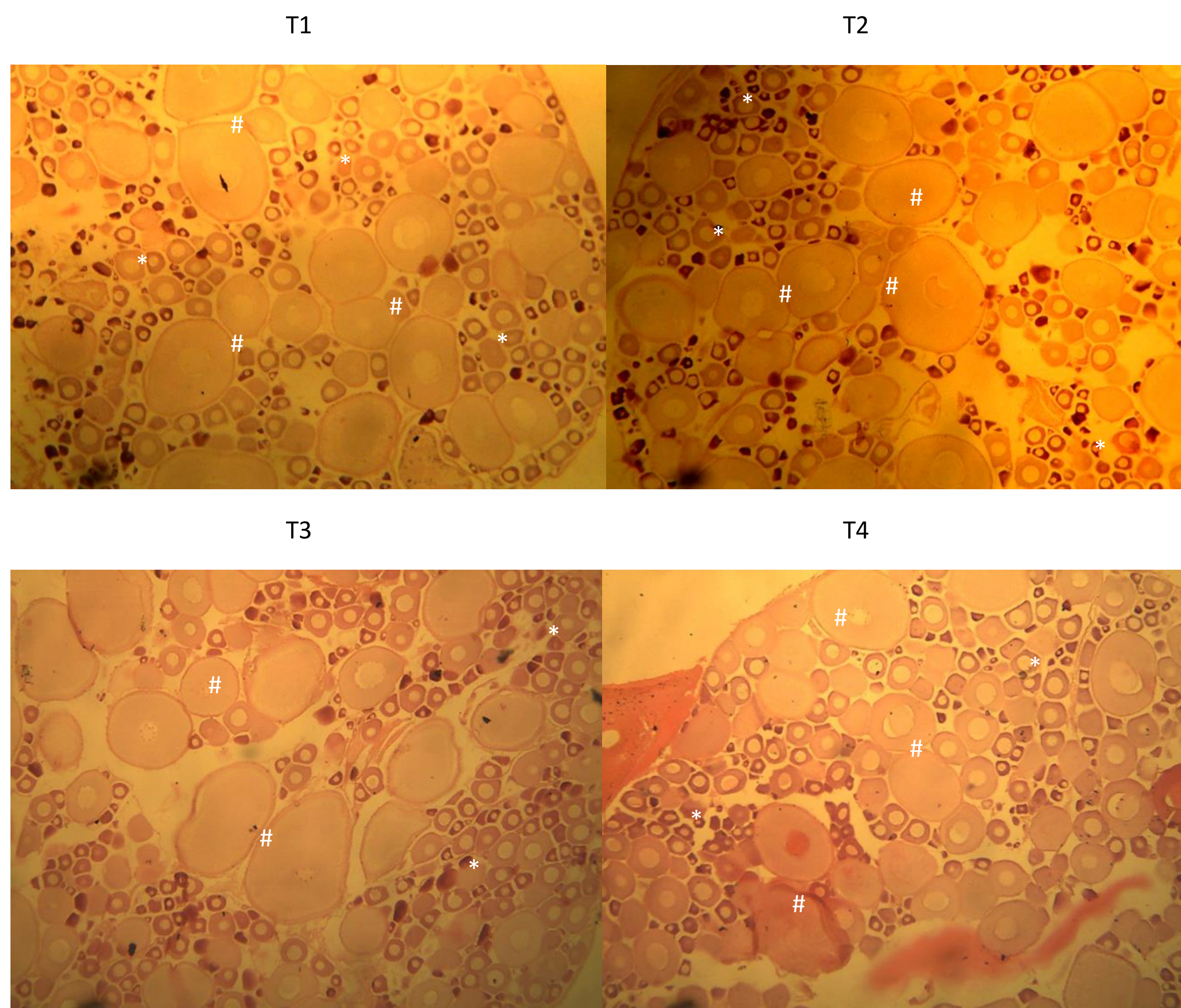
Oocyte histologic development was studied in all the experimental groups at 3rd, 5th, 7th and 9th week post treatment at the corresponding ages of 19, 21, 23, and 25 weeks with varying dietary inclusions of cocoa bean meal (CBM). At week 3 post-treatment (age of 19th weeks) ovaries of

fish in all experimental groups were similar. Oocytes in stages 1, 2, 3, and 4 were predominantly present while a few stage 5 oocytes were also seen. At week 5 of post-dietary treatment (21st week of age), ovaries of fish in T3, T4, and T5 were similar to those of the control group. They were seen to contain substantial numbers of oocytes in their secondary growth phase (stages 2, 3, and 4 of oocyte development) in addition to stages 1, 2, 3, 4, and 5 oocytes. Fish in group 2 (10% CBM) appeared to be more advanced in development as they were shown to contain a few population of oocytes in the maturation stage. At week 7 of dietary treatment (23rd week of age), oocytes in their secondary growth phase (SGP) were predominantly present in the ovaries of fish in the T5 (50% CBM) and the control group. Oocytes in their maturation phase were few in the ovaries of the fish in T3 (20% CBM) and T4 (40% CBM) but predominated in those of the T2 fish. During the last week of the experiment (10th week post treatment and 25th weeks of age) a similar differential pattern was observed for the oocyte distribution in the different groups. While those of the control groups had ovaries with predominant SGP follicles, those of the T2, T3, T4, and T5 had predominantly maturation phase (stage 6). However, the ovaries of the fish in group 2 (10%) showed sparse population of oocytes in the SGP.

The experimental animals used for the study were observed to have a group synchronous type of ovarian development. In a group synchronous ovarian development, at least two cohorts of oocytes can be distinguished in the maturing ovary; a fairly synchronous population of larger oocyte known as the clutch; and a more heterogeneous population of smaller oocytes from which the clutch is recruited. The result of this study collaborates the reports of Cek and Yilmaz (2007) and Saka and Adeyemo (2015) who showed that the ovary of *C. gariepinus* catfish at maturation is mainly occupied by a synchronous population of larger oocytes and a more heterogeneous population of pre-vitellogenic and vitellogenic oocytes detected among mature oocytes from which the clutch were recruited. It was observed that at the 9th week of post-treatment period (6 months of age), fish in the control group were made of predominantly pre-vitellogenic (SGP) oocytes while those of T2 (10% CBM) were relatively mature. This may be attributed to the polyphenolic constituent of the cocoa bean meal. According to Ly *et al*. (2014), sufficient maternal antioxidant status may help prevent and/or manage adverse mechanisms intimately related to poor reproductive performances and that are also associated with poor dietary habits and oxidative stress. This similar to the reports of Cek and Yilmaz (2007) who reported that wild *C. gariepinus* mature sexually at 1 year under laboratory conditions. However, the result was contrary to those of Schulz *et al*. (1994), Bail (1996), Cevaco *et al*. (1997), and Saka and

Adeyemo (2015) who reported that this species mature at 5 months post hatching. Early maturation of fish has been achieved either by genetic selection or better nutrition, revealing a correlation between maturation and growth. This explains the faster development/maturation observed for the fish in T2. There is scarcity of information as regards the effect of cocoa and its products on the gonadal development and fertility of fish. However, similar to the results of this study was shown that cocoa pod husk meal could be included in the diets of catfish up to 45% (that is 0.68g theobromine) without any adverse effect on growth and performance (Fagbenro and Sydenham, 1988; Fagbenro, 1995) and Nile tilapia (Pouomogne *et al.,* 1997). Abiola and Tewe (1991) reported an increase in egg weight and percentage egg production when CPM was fed to laying hens. However feeding cocoa powder or theobromine (up to 75mg/kg body weight) to rabbits from day 6 to 29 of gestation did not affect foetal weight and other reproductive performances (Tarka *et al.*, 1986). Contrary to this, theobromine which is high in cocoa powder has been reported to negatively affect reproductive health in mammals. It was shown that up to 250 mg/kg body weight administration altered testicular structure and affected spermatogenesis in mice (Wang *et al.*, 1992; Maleyki and Ismail, 2008). The faster maturation and development observed for the fish in group 2 with 10% CBM could be attributed to the antioxidant and pro-fertility properties of cocoa. Cocoa powder has been reported to contain up to 6% flavanols (mainly catechins and epicatechins) by weight in addition its high theobromine and modest content of protein, fat, and essential minerals such as copper and magnesium (DFI, 2003; Keen *et al.*, 2005). Polyphenols and methylxanthines exert antioxidant activities and immune protective ability under physiological conditions (Lee, 2000; Visioli *et al.*, 2000). These polyphenols have been shown to increase cellular lipid antioxidant activity of vitamin C; protects reactive oxygen species-induced degradation of lipids, proteins and 2-deoxyribose; and up regulates superoxide dismutase (Costa *et al.*, 2007; Intra and Kuo, 2007; Raza and John, 2007). This findings was supported by Callebaut (2008) who reported the use of chocolate for treatment of all sorts of ailments and diseases including fertility and depression.

WEEK 3

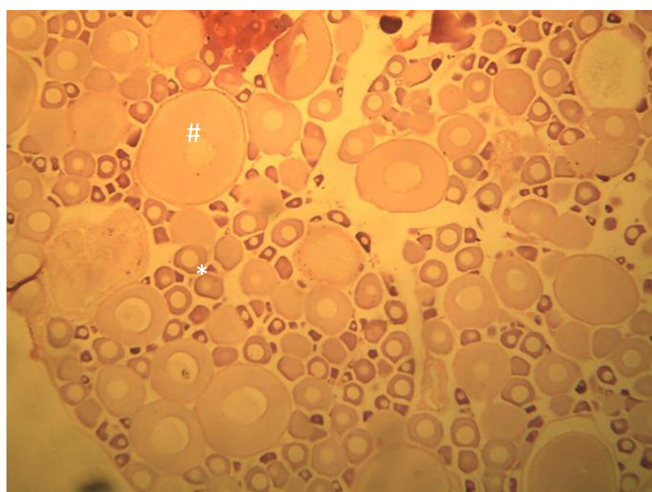


T5

Plate 17: Photomicrographs of the ovary collected from the different treatment groups at the end of week 3 showing predominance of oocytes in the primary and secondary growth phases of development. H&Ex100

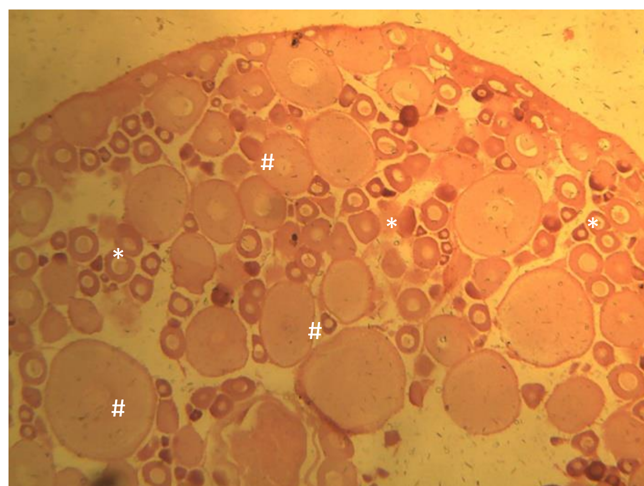
*- Area of high Primary growth phase oocytes population

#- Area of high Secondary growth phase oocytes population

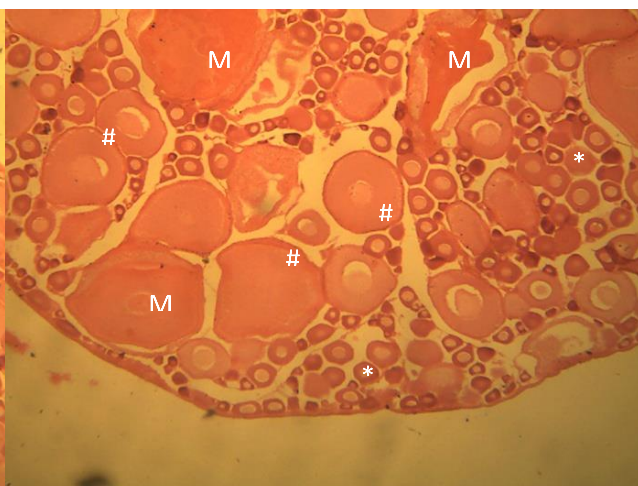


WEEK 5

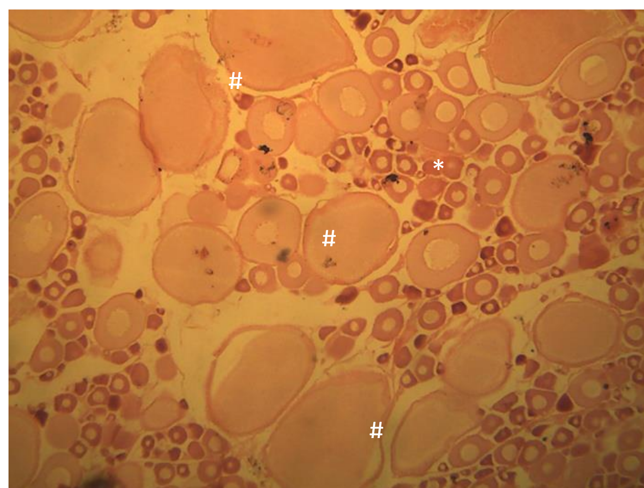
T1



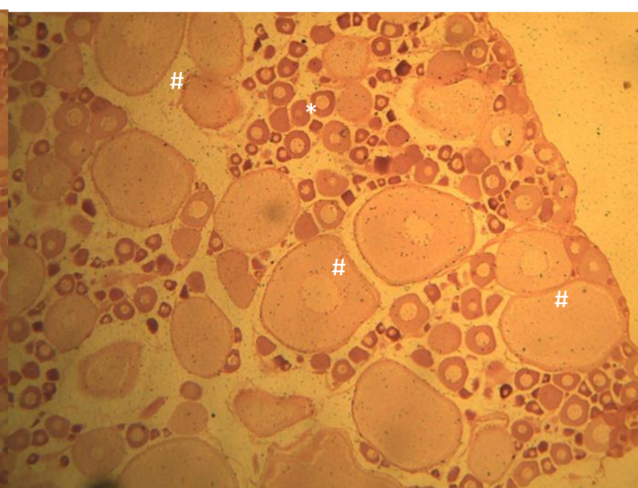
T2



T3

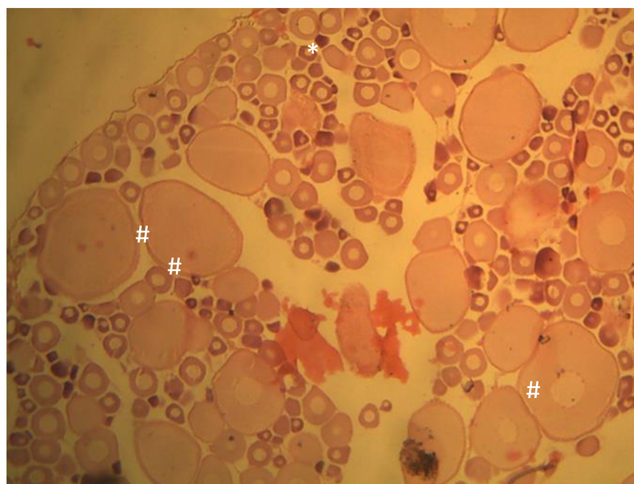


T4



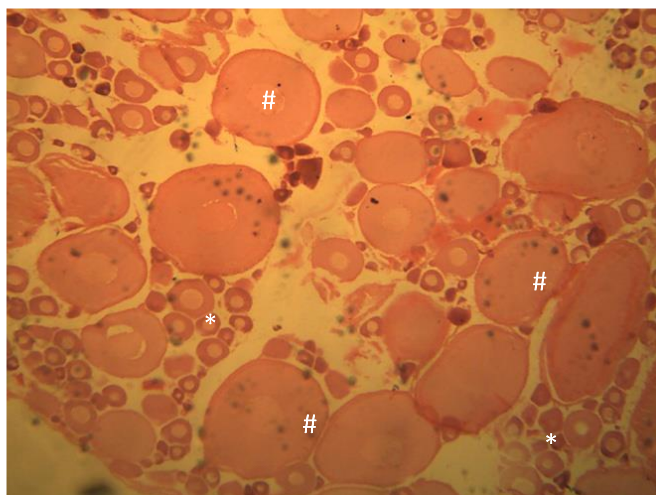
T5

Plate 18: Photomicrographs of the ovary collected from the different treatment groups at the end of

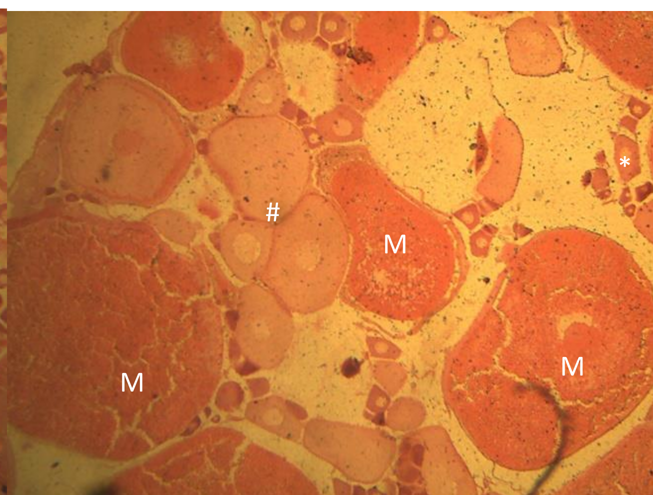


WEEK 7

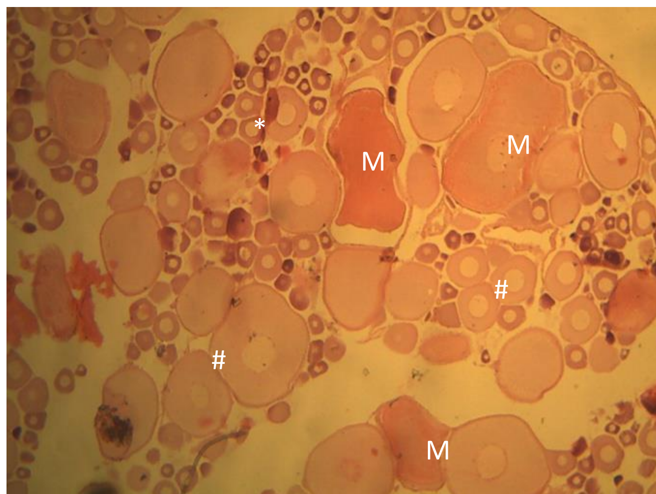
T1



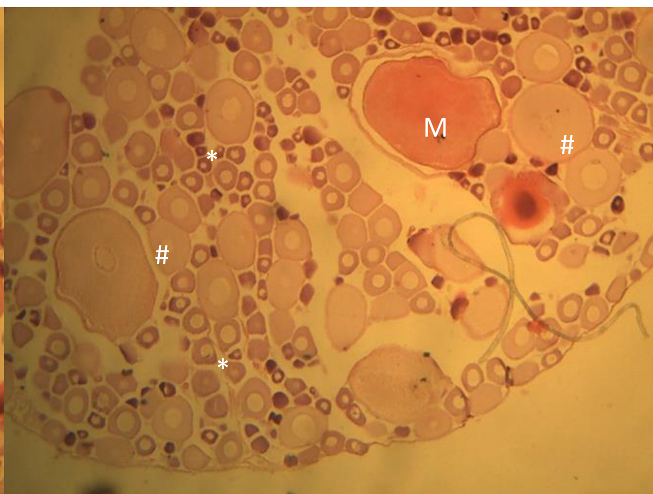
T2



T3



T4



T5

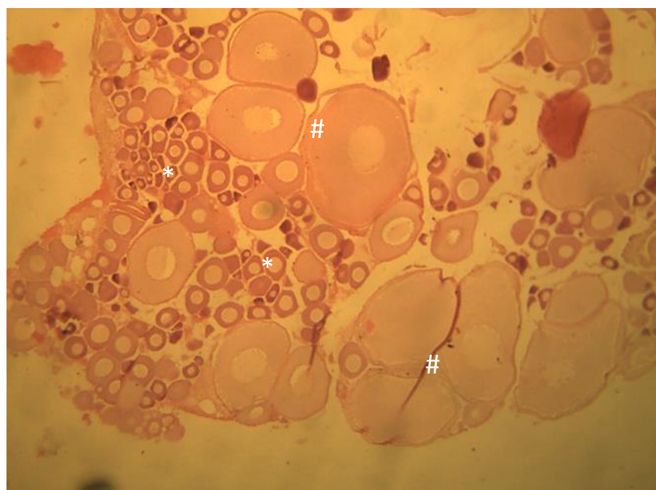
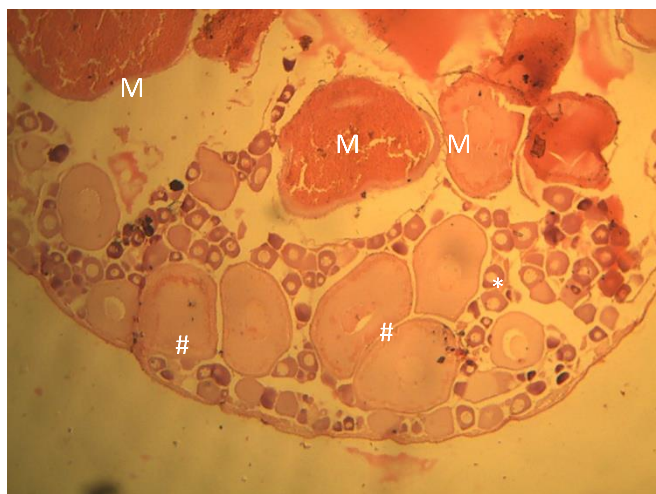


Plate 19: Photomicrographs of the ovary collected from the different treatment groups at the end of week 7 showing oocytes in the primary growth phase, secondary growth phase and maturation phases of oocyte development. H&Ex100

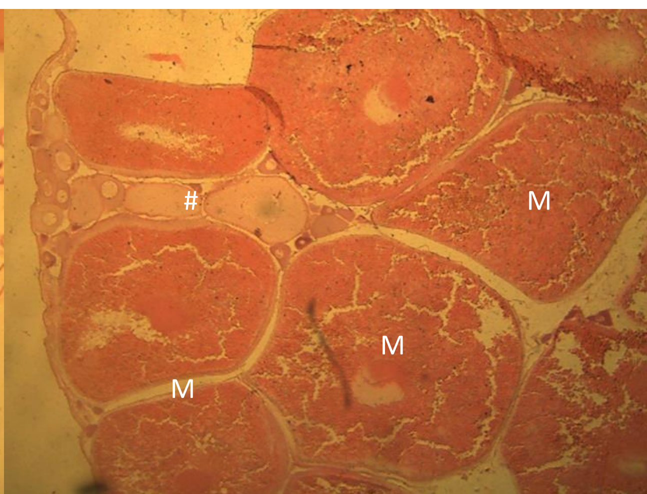
*- Area of high Primary growth phase oocytes
#- Area of high Secondary growth phase oocytes
M – Oocytes in the Maturation phase

WEEK 9

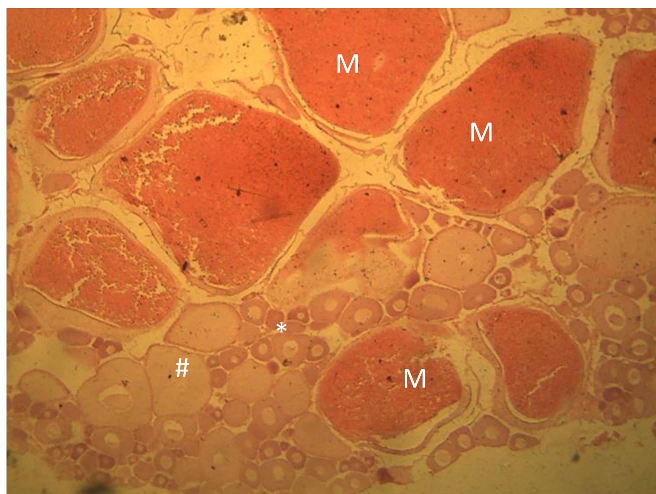
T1



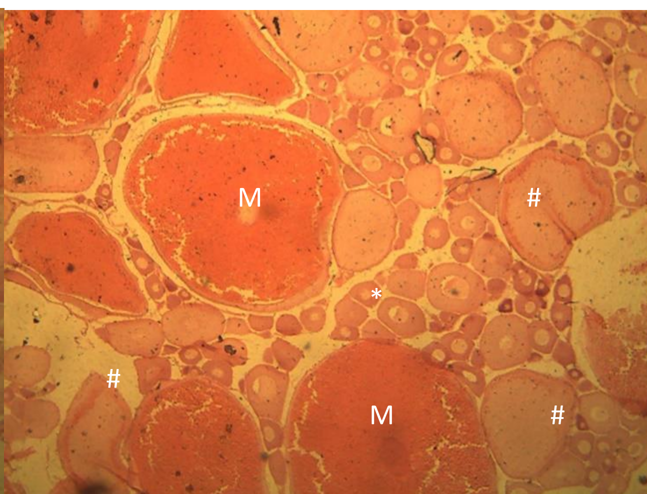
T2



T3



T4



T5

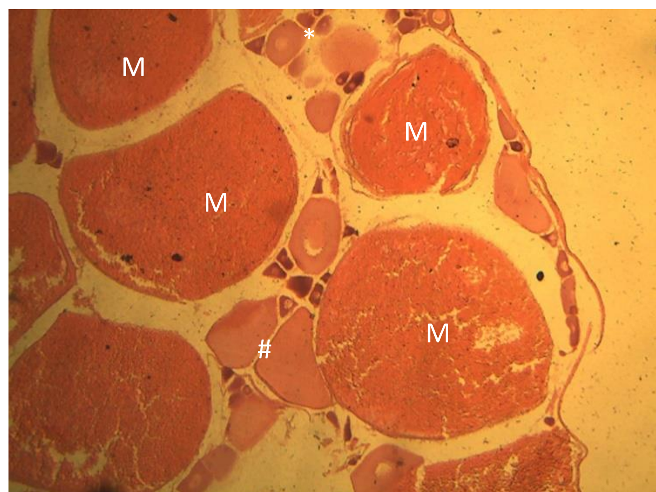


Plate 20: Photomicrographs of the ovary collected from the different treatment groups at the end of week 9 showing oocytes in the primary growth phase, secondary growth phase and maturation phases of oocyte development. H&Ex100

*- Area of high Primary growth phase oocytes
#- Area of high Secondary growth phase oocytes
M – Oocytes in the Maturation phase

4.2.5 Histomorphometry

The result of histomorphometric evaluations (oocyte diameter) of the different stages of oocyte development are represented in the Table 20.

Table 21: Means of oocyte diameters of the different developmental stages at weeks 3, 5, 7 and 9 post treatment

WEEKS	OOCYTE STAGE	T1 (0%) Mm	T2 (10%) Mm	T3 (20%) mm	T4 (40%) mm	T5 (50%) Mm	SEM
Week 3	S1	0.131	0.127	0.127	0.132	0.125	0.002^{NS}
	S2	0.246	0.262	0.288	0.246	0.279	0.004^{NS}
	S3	0.510	0.516	0.535	0.524	0.542	0.006^{NS}
	S4	0.789	0.800	0.824	0.807	0.818	0.009^{NS}
	S5	**1.109**^{b}	**1.187**^{a}	**1.152**^{a}	**1.094**^{b}	**1.094**^{b}	**0.007***
	S6	0.00	0.00	0.00	0.00	0.00	0.000^{NS}
Week 5	S1	0.127	0.129	0.127	0.124	0.133	0.020^{NS}
	S2	0.269	0.284	0.278	0.263	0.266	0.003^{NS}
	S3	0.531	0.548	0.567	0.543	0.543	0.006^{NS}
	S4	**0.847**^{b}	**0.983**^{a}	**0.835**^{b}	**0.802**^{b}	**0.847**^{b}	**0.012***
	S5	**1.118**^{c}	**1.287**^{a}	**1.180**^{b}	**1.128**^{bc}	**1.152**^{bc}	**0.011***
	S6	**0.0**^{b}	**1.806**^{a}	**0.0**^{b}	**0.0**^{b}	**0.0**^{b}	**0.085***
Week 7	S1	0.139	0.132	0.129	0.129	0.132	0.002^{NS}
	S2	0.293	0.288	0.266	0.267	0.277	0.004^{NS}
	S3	0.551	0.526	0.540	0.522	0.509	0.006^{NS}
	S4	**0.919**^{ab}	**0.949**^{a}	**0.876**^{bc}	**0.824**^{cd}	**0.812**^{d}	**0.010***
	S5	**1.169**^{b}	**1 .204**^{a}	**1.197**^{a}	**1.116**^{c}	**1.102**^{c}	**0.007***
	S6	**0.00**^{c}	**1.299**^{a}	**1.299**^{a}	**1.263**^{b}	**0.00**^{c}	**0.073***
Week 9	S1	0.132	0.129	0.127	0.126	0.138	0.002^{NS}
	S2	0.298	0.284	0.305	0.279	0.303	0.004^{NS}
	S3	0.564	0.550	0.538	0.546	0.540	0.005^{NS}

S4	**0.928^{b}**	**0.980^{a}**	**0.955^{a}**	**0.828^{c}**	**0.878^{c}**	**0.013^{*}**
S5	**1.238^{bc}**	**1.330^{a}**	**1.288^{ab}**	**1.193^{c}**	**1.182^{c}**	**0.012^{*}**
S6	**1.295^{b}**	**2.068^{a}**	**2.025^{a}**	**1.949^{a}**	**2.098^{a}**	**0.042^{*}**

$^{abc:}$ Row means with different superscript are significantly different at P<0.05; NS: non-significant; **S1: stage 1; S2: stage 2; S3: stage 3; S4: stage 4; S5: stage 5; S6: stage 6**

From the study, the means of the oocyte diameters in the primary growth phases (PGP) did not show any significant difference among treatment groups. However, significant differences were observed in the diameters of the oocytes in the secondary growth phase and maturation phase among the groups.

At week 3, the mean diameter of the stage 4 oocyte did not differ significantly between the treatment groups. The mean diameter of the stage 5 oocyte in groups T2 (10% CBM) and T3 (20% CBM) did not differ significantly from each other but showed significant increases (P<0.05) when compared to the means of the stage 5 oocyte diameters of the other groups (T1, T4, and T5).

At week 5, oocytes at stage 6 developmental were observed only in group T2 (10% CBM). The mean diameters of the stage 4, 5 and 6 oocytes in group T2 (10% CBM) showed significant (P<0.05) increases when compared to those of the other groups. Also, the means of stage 4, 5, and 6 oocytes did not differ significantly (P>0.05) among T4, T5, and T6.

At week 7, oocytes of all developmental stages were observed in groups T2 (10% CBM), T3 (20% CBM) and T4 (40% CBM) while groups T1 (0% CBM) and T5 (50% CBM) showed only oocytes in the Primary growth phase (PGP) and Secondary growth phase (SGP) with no stage 6 oocytes. The means of the stage 4 oocytes diameters in group T2 (10% CBM) showed a significant increase, compared to means of the stage 4 oocytes in the other groups. Likewise, the means of the diameters of the oocytes in stage 5 of maturation showed significant increases in groups T2 (10% CBM) and T3 (20% CBM), compared to those of the other groups. The mean diameters of the oocytes in the maturation phase (stage 6) in groups T2 (10% CBM) and T3 (20% CBM) did not differ significantly from each other. However, they showed a significant (P<0.05) increases in diameter compared to oocytes of group T4 (40 % CBM).

At week 9, all the oocytes developmental stages were observed in all the groups. The mean diameters of the stage 4 oocyte showed significant (P<0.05) increase in groups T2 (10% CBM) and T3 (20% CBM) compared to the other groups. The mean diameter of the stage 5 oocytes showed significant increases in groups T1 (0% CBM), T2 (20%) and T3 (40%), compared to

groups T4 (40% CBM) and T5 (50% CBM). Finally, the mean of the stage 6 oocyte diameters showed significant ($P<0.05$) increases in T2, T3 and T5 groups compared to T1 and T4 groups.

In this study, the oocyte diameters recorded for the different stages of oocyte development in the untreated control group (T1) showed varying range of values which were similar to those observed in groups T4 and T5 treated with 40% and 50% cocoa bean meal. Groups T2 and T3 treated with 10% CB and 20% CB respectively, showed varying increases in the sizes of the oocytes in the secondary growth phase and maturation phases. Out of the two, T2 (10% CBM) showed the highest values. This larger oocyte diameter observed for the ovaries of the fish in T2 (10%) suggest the enhanced antioxidant and pro-fertility potentials of cocoa powder. At this level of inclusion, the antioxidant properties of cocoa arises from its high content of polyphenols (catechin and procyanidin) (Wan *et al.*, 2001). Polyphenols have been reported to act as proton donor, scavenging radicals and inhibiting enzymes that increases oxidative stress (Rice-Evans *et al.*, 1997; Helm *et al.*, 2002). Also, flavonoids and procyanidins were found to prevent lipid oxidation through interaction between lipid membranes and the adsorption to the polar lipid head groups (Verstraeten *et al.*, 2005). These properties enable them to act as anticarcinogenic, antiinflammatory, antihepatotoxic, antibacterial, antiviral, and antiallergenic c ompounds (Arts and Hollman, 2005; Vita, 2005) and other promising health effects (Maleyki and Ismail, 2008). Also, cocoa has been known to contain a large amount of theobromine (2 to 3% in dry form) which has been reported to negatively affect reproductive health. The non-significant ($P\times0.05$) differences observed for the fish in T4 and T5 when compared to those of the control and T2 could possibly be attributed to theobromine which have been reported to be high in cocoa products which may have shielded the effect of the beneficial polyphenols.

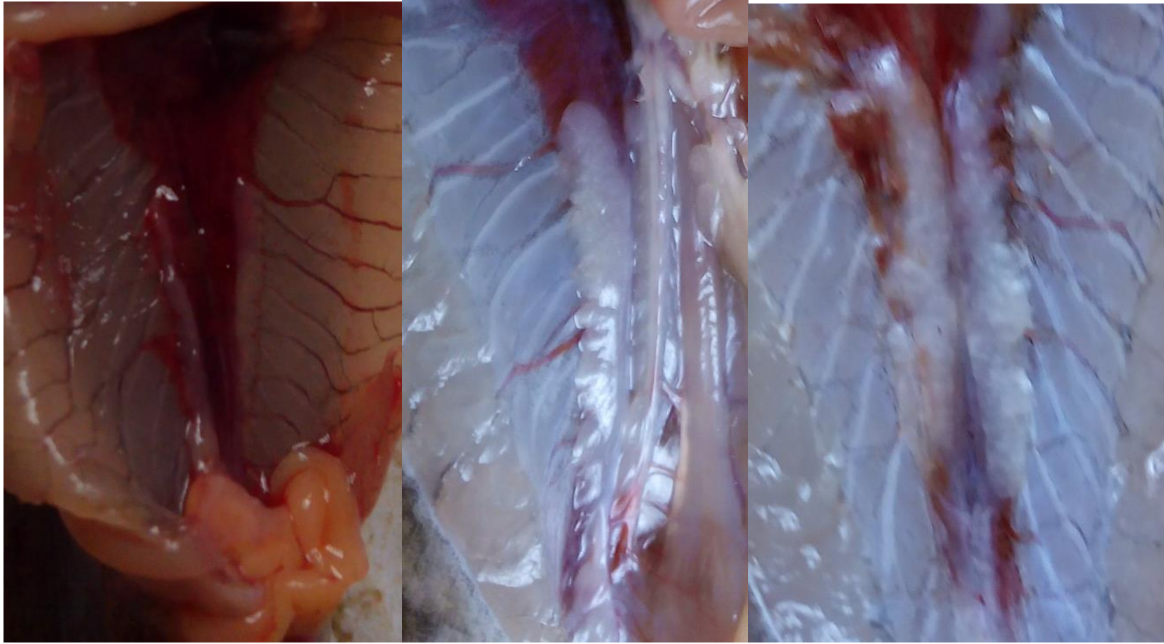
4.3 TESTICULAR DEVELOPMENT

4.3.1 Description and classification of the testes into stages

The stages of testicular development were characterized based on gross morphology according to the descriptions reported by Saka *et al*. (2015). Only stages I and II testes were observed during the course of the study. Stages III and IV testes were not observed during the course of the study. The stages observed are as follows.

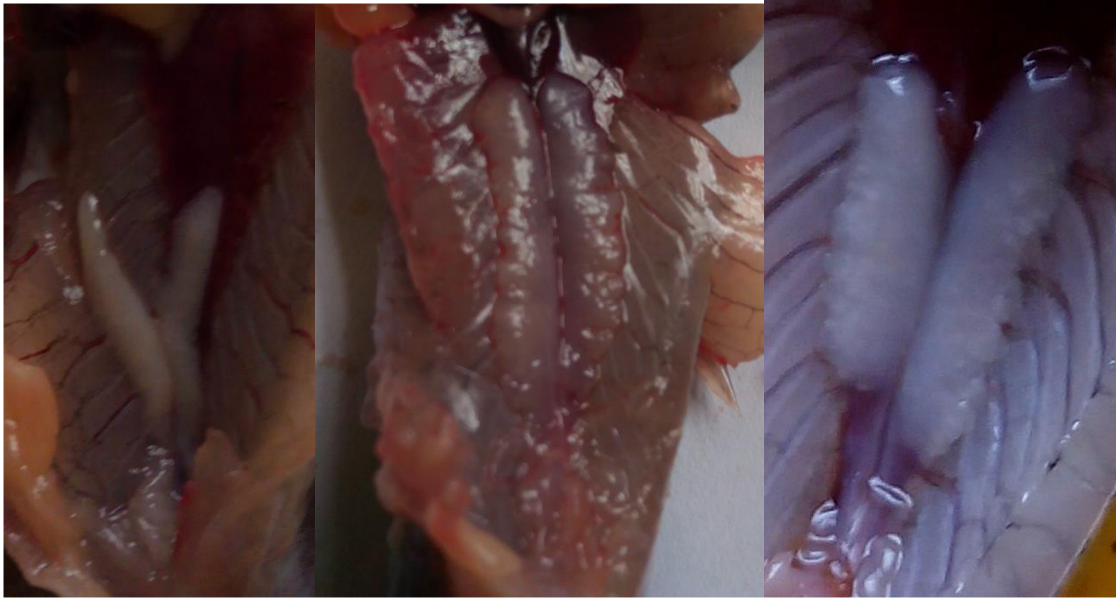
Stage I: The testes were generally small and translucent. They were distinguished from the stage I ovary by their thinner and longer size. These were seen at the onset of the study (at their 15 weeks of age).

Plate 21: Gross morphology of stage I testes in *C. gariepinus* catfish



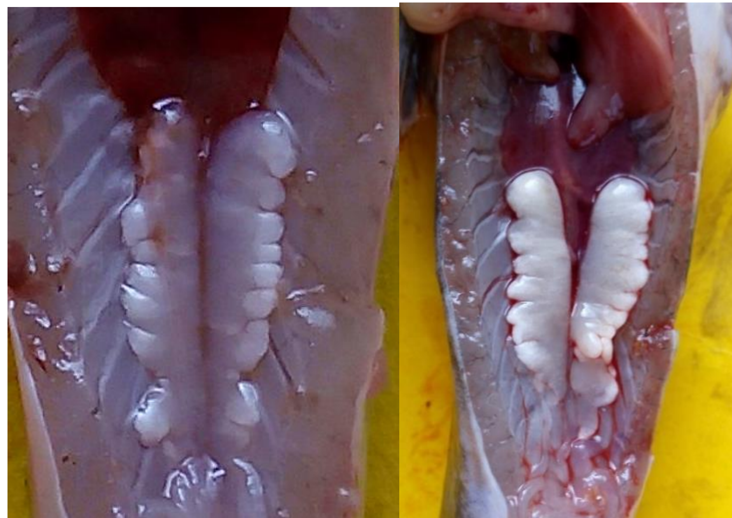
Stage II: Stage II testes were whitish to pinkish in colour and progressively increased in size as the fish mature and age. Milt was not presence in sperm duct or was not voided when the testes was squeezed. These were seen at about 15 weeks of age (also at about the onset of the experiment). A pictograph of the group II testes are shown in the plate 22.

Plate 22: Gross morphology of stage II testes in *C. gariepinus* catfish



Early stage III: Testes were larger, fuller and well developed. Some part of the testes were whitish in colour. These were seen at the 24th week that is 6 months of age (10th week of sampling).

Figure 23: Gross morphology of stage III testes in *C. gariepinus* catfish



4.3.2 Effect of varying dietary levels of cocoa bean meal on testicular development of *C. gariepinus* catfish.

The results of the body weight and testicular growth and development of *C. gariepinus* catfish fed diet with varying levels of cocoa bean meal are presented in Table 21.

Table 22: Effect of varying dietary levels of cocoa bean meal on body weight and testicular growth *C. gariepinus* catfish

Weeks	Indices	T1 (Control)	T2 (10% CBM)	T3 (20% CBM)	T4 (40% CBM)	T5 (50% CBM)	SEM
3	B. W	17.08	17.67	15.31	14.51	14.39	1.75^{NS}
	T. W	0.034	0.0638	0.0238	0.0422	0.0153	0.0983^{NS}
	GSI	0.202	0.4088	0.1578	0.2213	0.1225	0.6036^{NS}
5	B. W	18.49^{ab}	20.717^{ab}	22.10^{a}	19.61^{ab}	15.89^{b}	1.27*
	T. W	0.042^{ab}	0.0735^{a}	0.0497^{ab}	0.057^{ab}	0.02^{4b}	0.0058*
	GSI	0.241^{ab}	0.353^{a}	0.217^{ab}	0.2753^{a}	0.153^{b}	0.0939*
7	B. W	21.84	21.50	23.14	25.24	18.80	1.37^{NS}
	T. W	0.0817	0.108	0.0663	0.0893	0.046	0.0105^{NS}
	GSI	0.291	0.4087	0.277	0.442	0.2387	0.0475^{NS}
9	B. W	27.81^{ab}	35.068^{a}	32.89^{ab}	37.665^{a}	22.06^{b}	1.869*
	T. W	0.0865^{ab}	0.2515^{a}	0.08^{ab}	0.2073^{a}	0.059^{b}	0.0279*
	GSI	0.387^{b}	0.6623^{a}	0.284^{b}	0.544^{a}	0.302^{b}	0.615*

^{abc} Row means with different superscripts are significantly ($P<0.05$) different. B. W.: Body weight; T.W.: Testicular weight; GSI: Gonadosomatic index; NS: Non-significant.

There were no significant (P×0.05) differences among the treatment groups in body weight, testes weight and the GSI in the 3rd and 7th weeks of the study. At the 5th week, B.W., T.W., and GSI significantly ($P<0.05$) differed among the treatment group. T3 had the highest B.W. value (22.01g) which was significantly ($P<0.05$) different from T5 which had the least value (15.89g). The T2, T4, and the control had values (20.72, 19.61, and 18.49g, respectively) which did not differ significantly (P×0.05) from those of T3 and T5. Also, T2 had the highest T.W. (0.0735g) and GSI (0.353) and was significantly ($P<0.05$) different from T5 which had the least T.W. and GSI values (0.024g and 0.153, respectively). There were no significant (P×0.05) difference in T.W. and GSI among T3, T4 and the control and they also did not differ significantly (P×0.05) from T2 and T5. At week 9 of the study, there were significant ($P<0.05$) differences in the B.W.,

T.W., and GSI values among the treatment groups. T2 had the highest T.W. and GSI values (0.2515g and 0.662 respectively). This was followed by T4 which had values (0.2073g and 0.0544 respectively) and they were not significantly (P×0.05) different from T2. The T2 and T4 groups were however significantly (P<0.05) different from T5 which had the least values in T.W. and GSI (0.059g and 0.302 respectively). T3 and the control had T.W. values (0.284 and 0.387g respectively) which were not significantly different from those of other treatments. On the other hand, T3 had the least GSI values which were not significantly different T5 (0.302) and the control (0.387). Testes weight ranged from 0.059g in T5 to 0.2515g in T2 while the GSI values ranged from 0.302 in T5 to 0.6623 in T2. The ranges of GSI values obtained for the male *C. gariepinus* catfish in this study are similar to those reported by Dada and Aguda (2015) (0.45 to 0.94) and Dada and Ejete-Iroh (2015) (0.36 to 0.69) in catfish *C. gariepinus*. These authors also reported testicular weight values of 3.12 to 6.83g.

Similarly, the gross morphology follows the same development pattern as was indicated by the T.W. and GSI. A pictograph showing the testes of the fish at weeks 5 and 9 are shown in Plates 24 to 30. As shown by the pictograph of the gross morphology of the fish testes in week 5 of the study, the testes of fish in T1, T2, T3, and T4 were observed to be similar in growth and development. They are classified according to the descriptive classification of Saka *et al*. (2015) as stage II testes. Testes of the fish in T5 were however, smaller than those of the other groups. They were seen as ribbon-like and tube-like structures, and were judged to still be in stage I of testicular development. At week 9 of the study, there were pronounced growth of the testes of the fish. The testes of the T2 and T4 fish appeared to be in the early stage III of testicular development of the male *C. gariepinus* catfish as described by Saka *et al*. (2015). Similar results were reported by Dada and Aguda (2015) using *T. conophorum* as a fertility enhancing agent for male catfish *C. gariepinus.* Also, Dada (2013) reported enhanced fertility in male catfish *C. gariepinus* using medicinal herb (*Sesame indicum*). Dada and Ogunduyile (2011) reported improved reproductive performance of male catfish *C. gariepinus* broodstocks fed diets supplemented with medicinal plants (*Mucuna pruriens*) when compared to those on the control diet. Furthermore, improved performance indicated in increased reproductive indices in male African catfish (*C. gariepinus*) fed dietary fluted pumpkin was reported by Dada and Ejete-Iroh (2015). The antioxidant effect of polyphenols are thought to be associated with their ability to stimulate the antioxidant defense metabolism through redox-regulated transcription factors and mitogen-activated protein kinase-dependent cell cycle regulation (Jiao *et al*., 2003; Williams *et*

al., 2004). The production of ROS is a normal physiological event in various organs including the testis. Overproduction of these ROSsø can be harmful to sperm and subsequently to male fertility **(**Akiyama, 1999). Cocoa powder has been reported to be very rich in polyphenols (flavonoids) which possess strong antioxidant effects in vivo hence could aid in amelioration oxidative damages. Cocoa powder is also known to be an excellent source of most essential minerals especially calcium, copper, iron, manganese, magnesium, phosphorus, potassium, and zinc (Rucker, 2009). Some of these minerals in addition to flavonoids have well documented spermatogenic activities. Zinc for instance promotes growth, sexual maturation and reproduction and there has been evidence associating serum and semen zinc levels with male infertility (Mohan *et al*., 1997). It could be concluded that addition of cocoa bean meal to the diets of catfish provided a steady supply of additional nutrients compared to the control. The adverse effect of cocoa bean meal reported at high levels of 50% could be attributed to the theobromine and some other anti-nutritional (tannin) component of this product which have been reported to negatively affect reproductive performace. This corroborates the reports of theobromine-induced alterations in sertoli cells and in testicular morphology (Wang *et al*., 1992)

The gross morphology of the testes of *C. gariepinus* catfish at week 5 and 9 are shown in Figures 26 and 27 respectively.

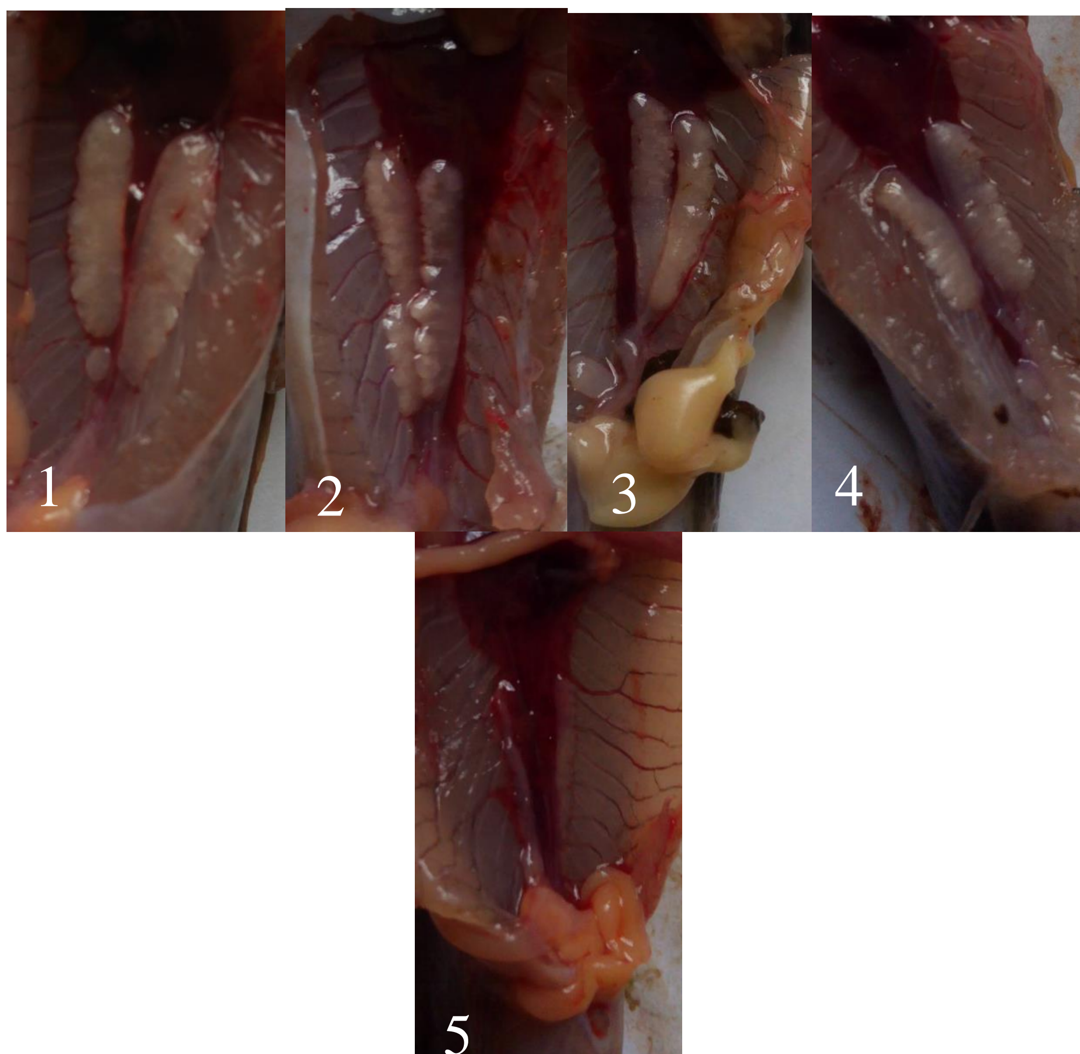


Plate 24: Pictograph of the gross morphology of the *C. gariepinus* catfish testes at week 5. 1, 2, 3, 4, and 5 represent treatments 1, 2, 3, 4, and 5 respectively. T1, T2, T3, and T4 testes are in similar in appearance and size. They are larger and more developed than T2 testis.

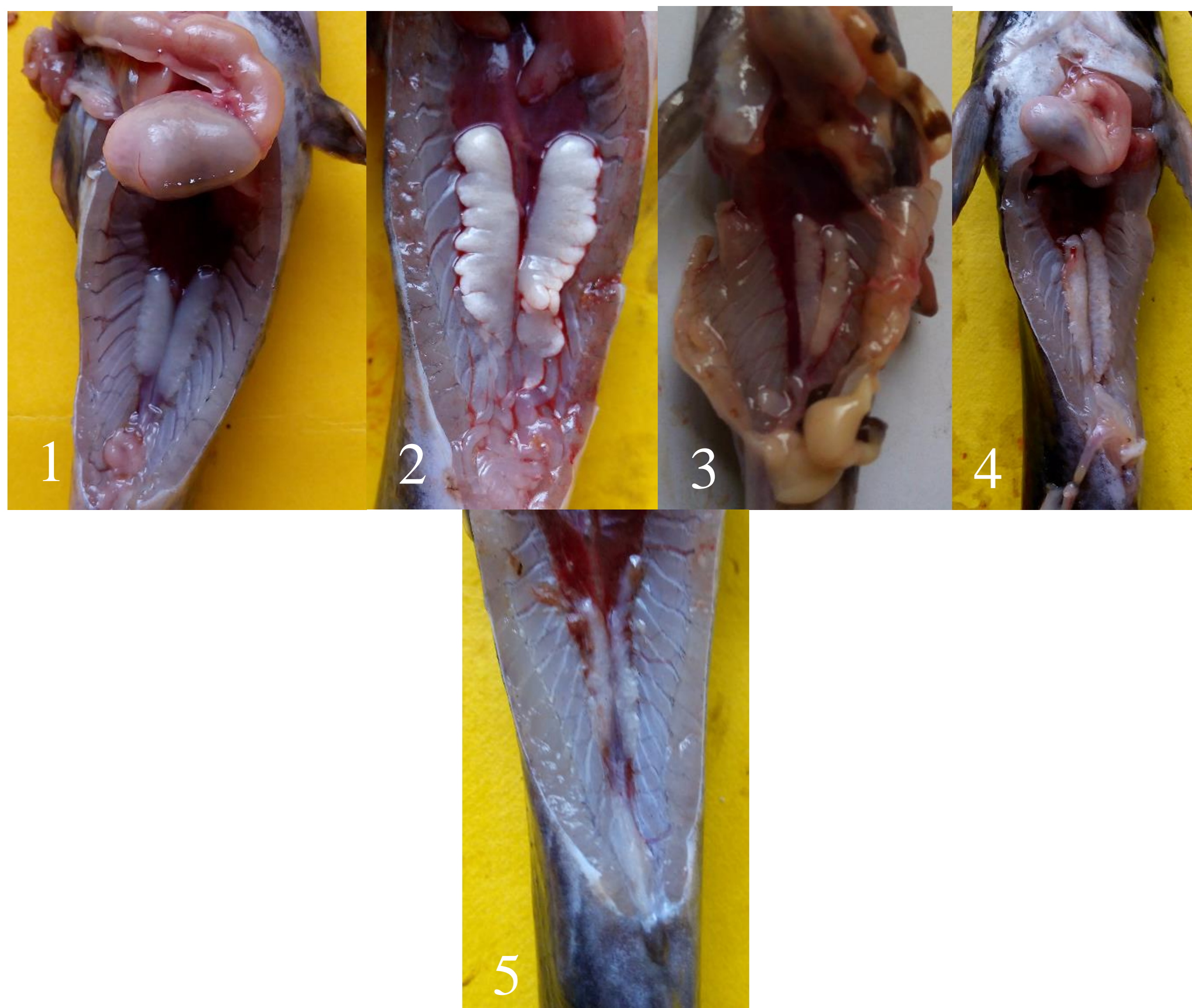


Plate 25: Pictograph of the gross morphology of the *C. gariepinus* catfish testes at week 9. 1, 2, 3, 4, and 5 represent treatments 1, 2, 3, 4, and 5 respectively. T2 and T4 are seen to be larger and more developed than other treatments and are classified as early stage III according to Saka *et al.* (2015).

4.3.3 Histomorphology of the *C. gariepinus* catfish testes

Tissue samples from each treatment group and their replicates were examined at weeks 3, 5, 7 and 9 of treatment, to determine their maturity stages. Four (4) histological stages have been defined according to the maturation stages of the testes in fishes (described by Saka *et al*, 2015). The histomorphology of the fish from the different treatment groups are presented in the Figures below.

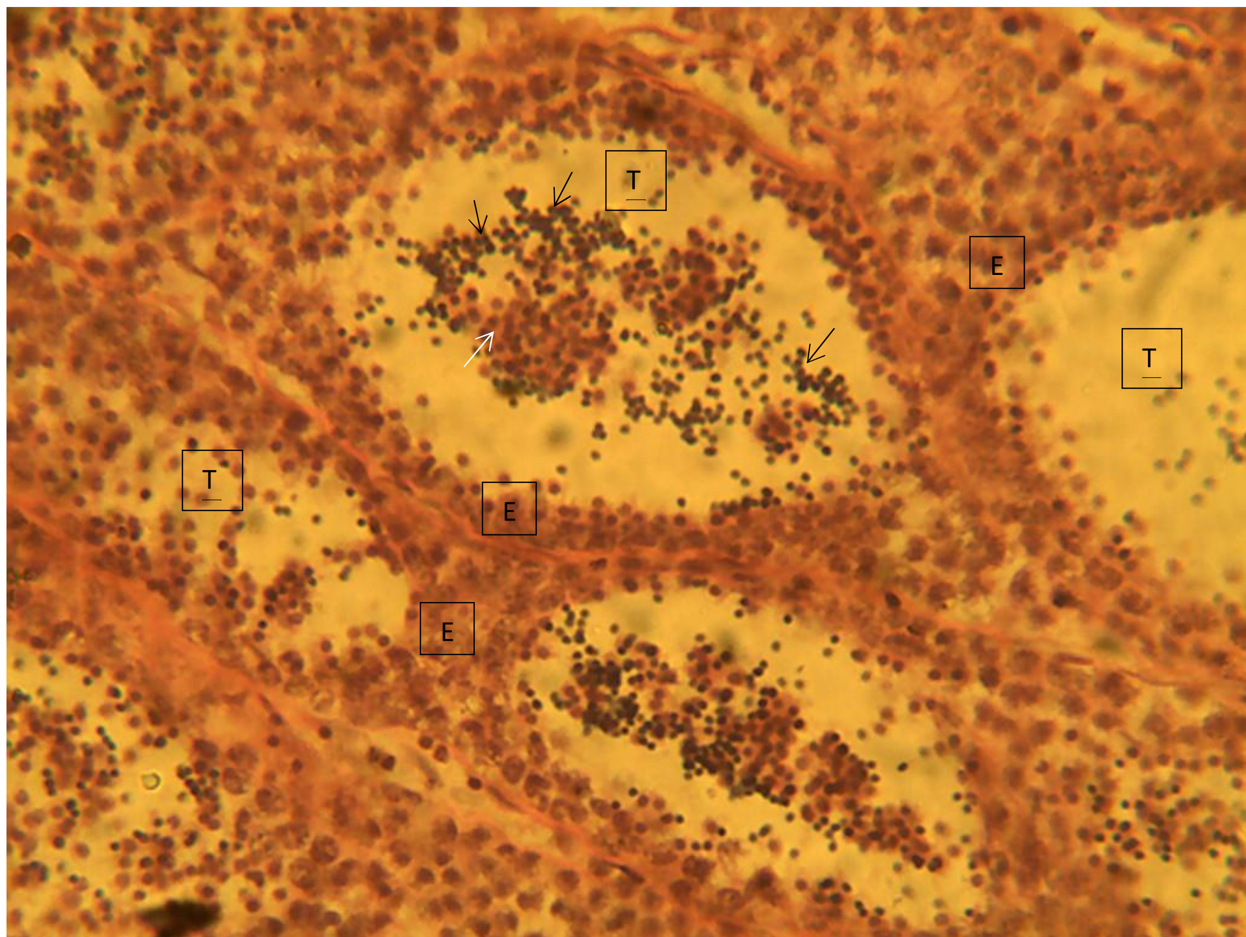


Plate 26: Photomicrograph of a section of the testes from group T1 at the termination of the experiment showing the normal histomorphology of the testes in the third stage of development. Note the band clusters of an admixture of the different germ cells making up the epithelium (E) of the seminiferous tubules. Inside the lumen of the seminiferous tubules (T) are clusters of spermatozoa (black arrow) and secondary spermatids (whiter arrow). H&Ex400

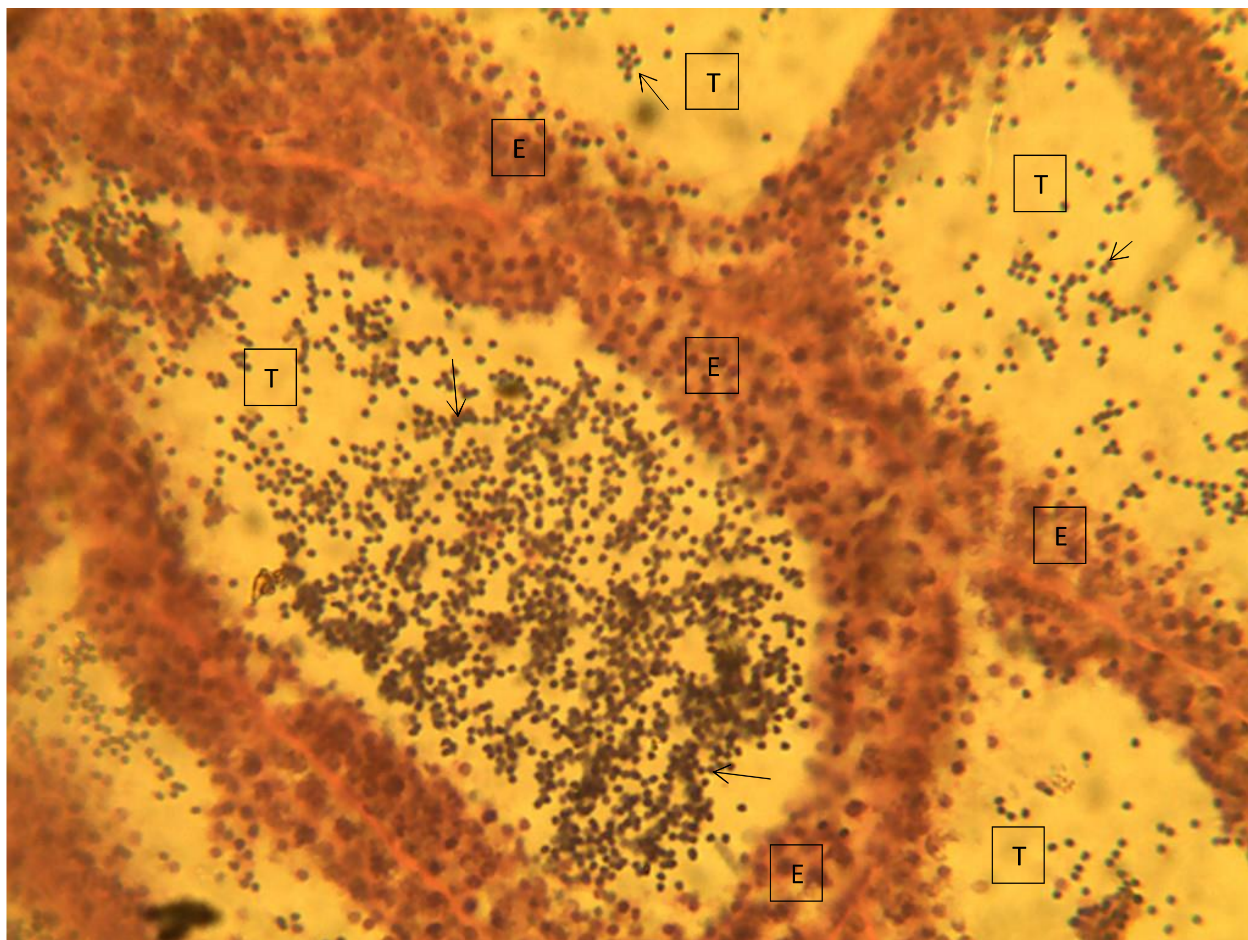


Plate 27: Photomicrograph of a section of the testes from group T2 at the end of the experiment showing the normal histomorphology of the testes in the third stage of development. Note the band clusters of an admixture of the different germ cells making up the epithelium (E) of the seminiferous tubules. Inside the lumen of the seminiferous tubules (T) are clusters of spermatozoa (black arrow) and secondary spermatids (white arrow). H&Ex400

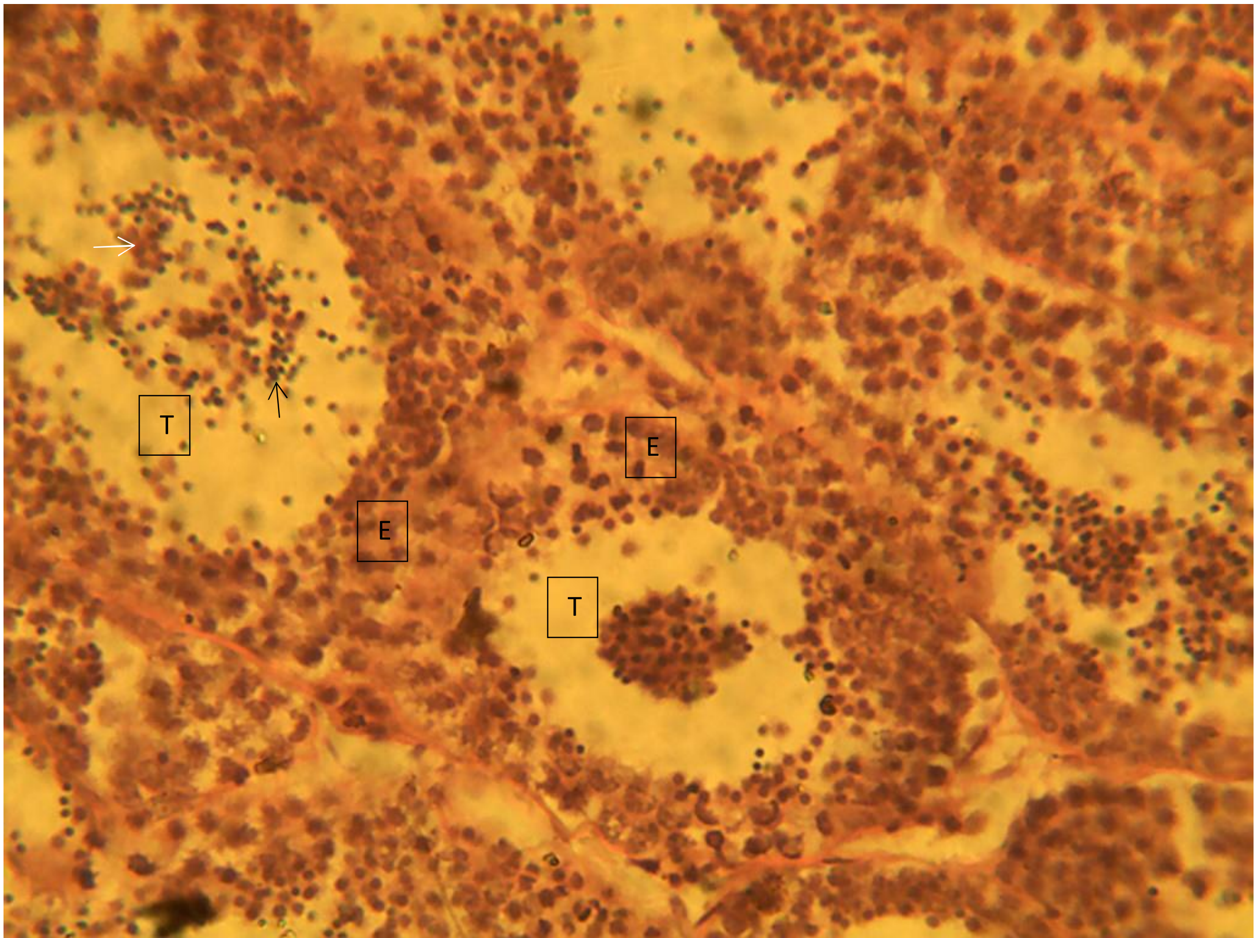


Plate 28: Photomicrograph of a section of the testes from group T3 at the end of the experiment showing the normal histomorphology of the testes in the third stage of development. Note the band clusters of an admixture of the different germ cells making up the epithelium (E) of the seminiferous tubules. Inside the lumen of the seminiferous tubules (T) are clusters of spermatozoa (black arrow) and secondary spermatids (white arrow). H&Ex400

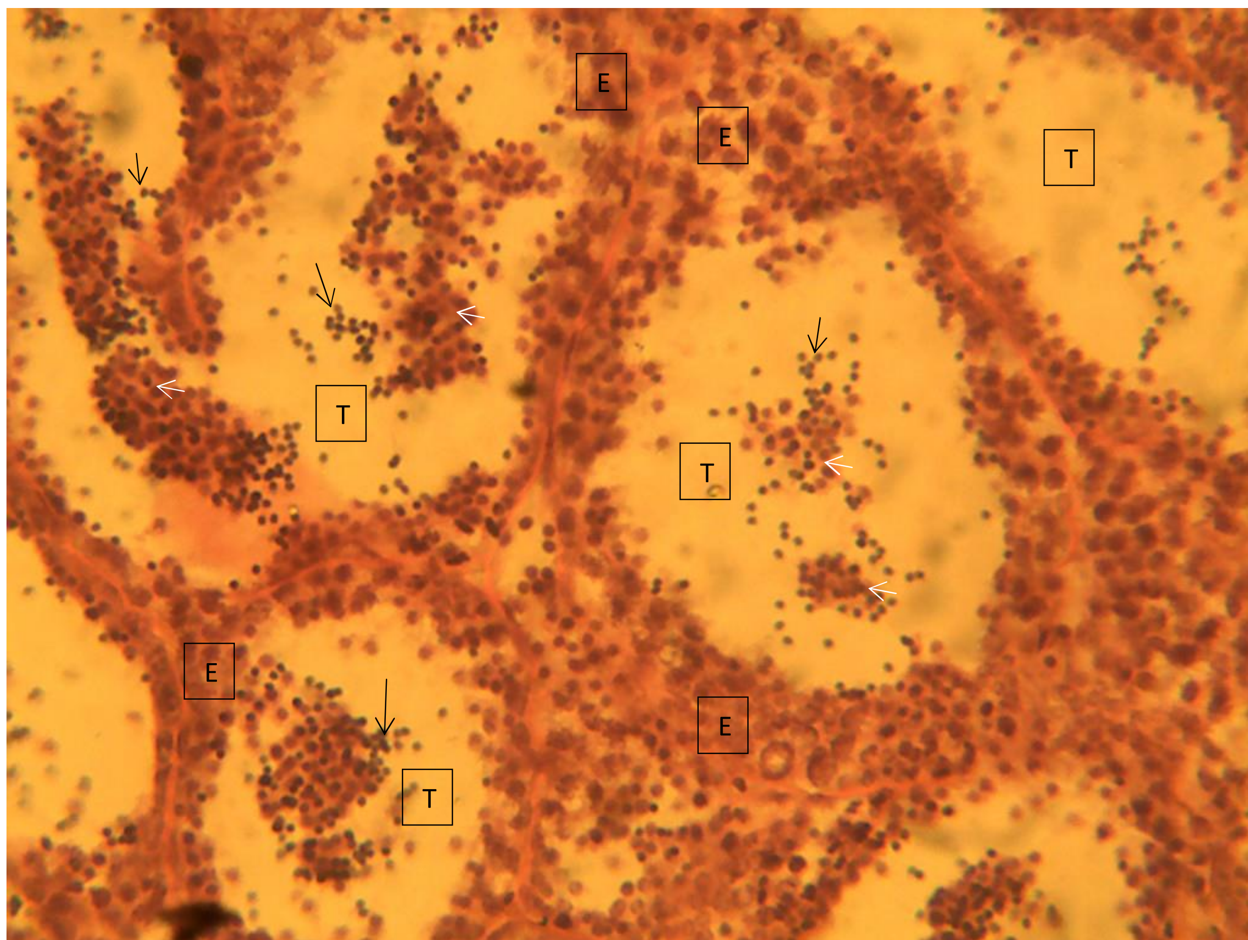


Plate 29: Photomicrograph of a section of the testes from group T4 at the end of the experiment showing the normal histomorphology of the testes in the third stage of development. Note the band clusters of an admixture of the different germ cells making up the epithelium (E) of the seminiferous tubules. Inside the lumen of the seminiferous tubules (T) are clusters of spermatozoa (black arrow) and secondary spermatids (white arrow). H&Ex400

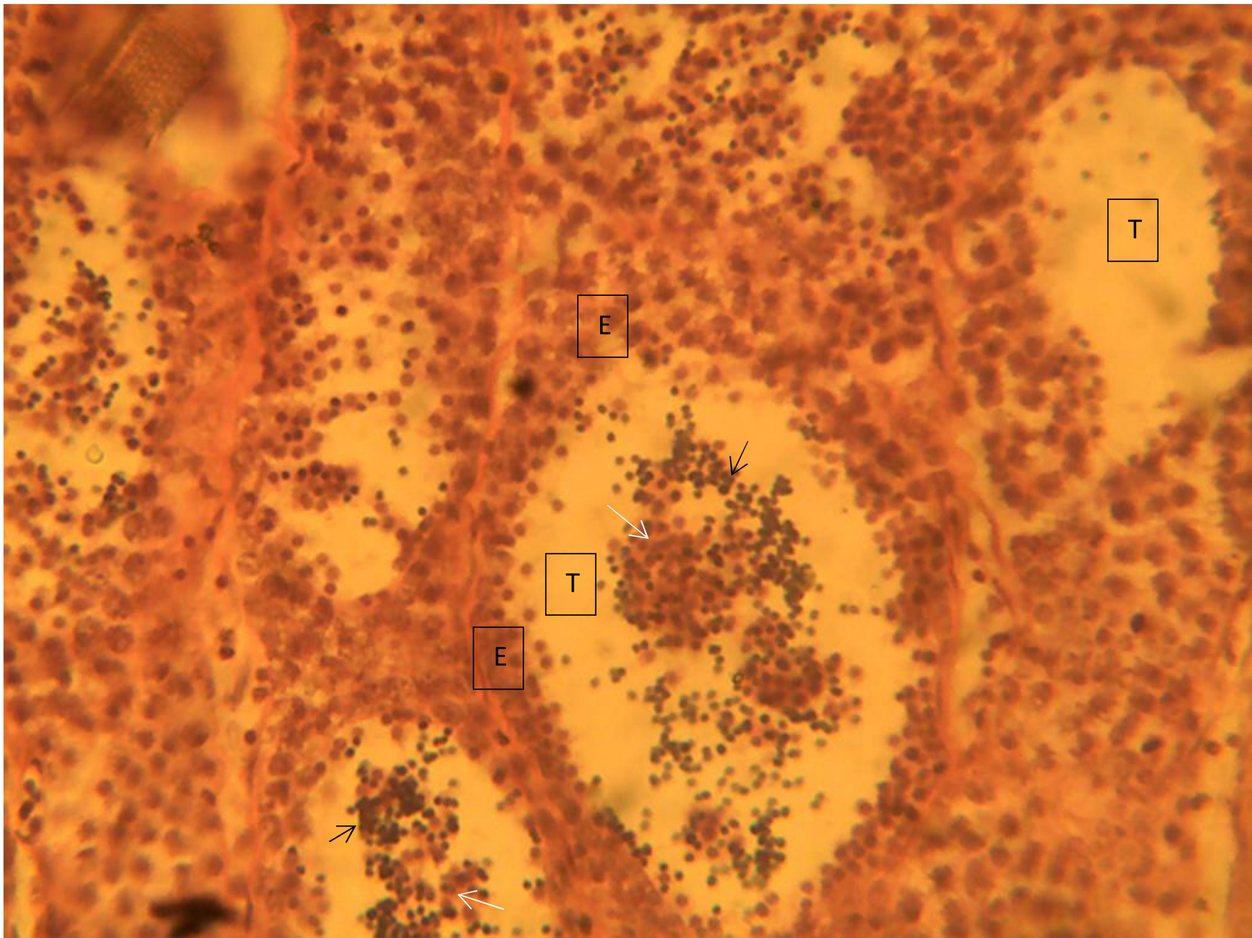


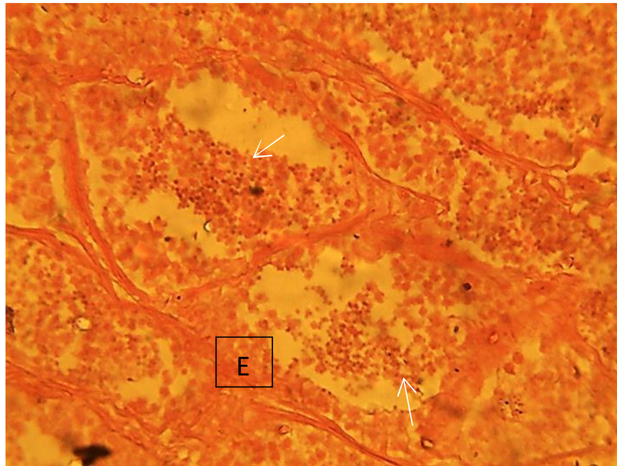
Plate 30: Photomicrograph of a section of the testes from group T5 at the end of the experiment showing the normal histomorphology of the testes in the third stage of development. Note the band clusters of an admixture of the different germ cells making up the epithelium (E) of the seminiferous tubules. Inside the lumen of the seminiferous tubules (T) are clusters of spermatozoa (black arrow) and secondary spermatids (white arrow). H&Ex400

In this study, no testicular histomorphological differences were observed between the different treatment groups at the 3rd, 5th, 7th, and 9th week of treatment. All the experimental group showed testicular histology which appear to be in the third stage of maturation. Examination of the histological sections showed a marked asynchronous development in the spermatogenesis within the same testis. The sections revealed round, oblong to irregularly shaped seminiferous tubules of varying dimensions. All developmental stages of the germ cells could be observed inside these seminiferous tubules. At the basement membrane of the tubules, the spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids form band clusters, making up the tubular epithelial height. The primary spermatocytes appear to be slightly bigger than the secondary spermatocytes, and have much denser and less-rounded nuclei. The spermatids are much smaller than the secondary spermatocytes, and have very dense and rounded nuclei. In the

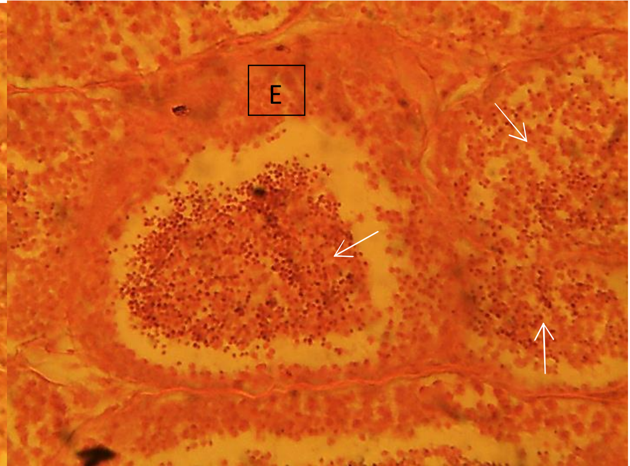
lumen of the seminiferous tubules, spermatozoa which have dense nuclei and inconspicuous cytoplasm are packed, filling the lumen. A few spermatids were also observed in the lumen.

WEEK 3

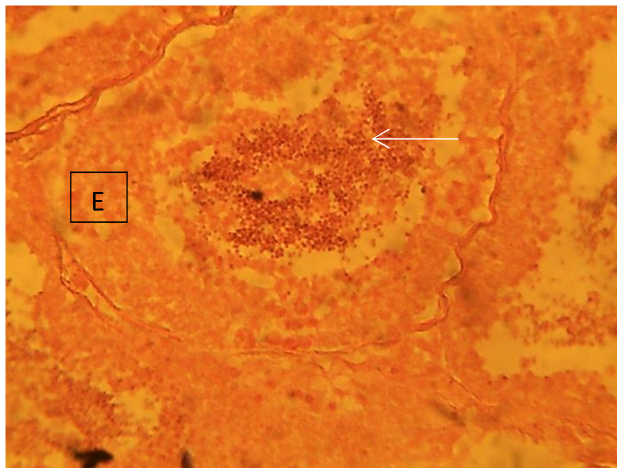
T1



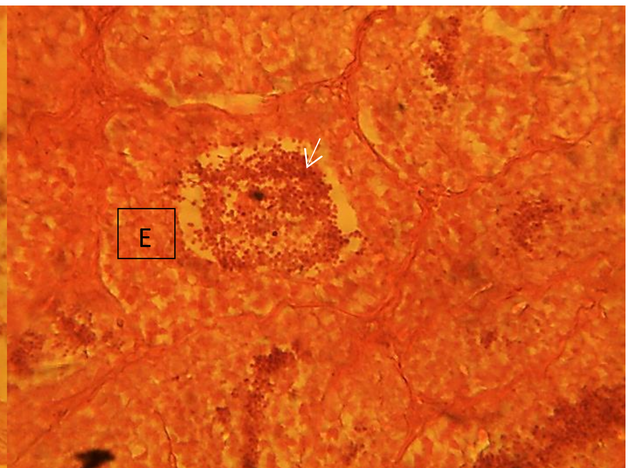
T2



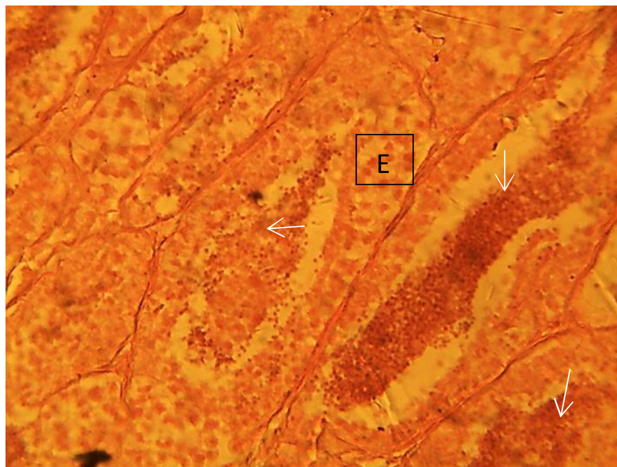
T3

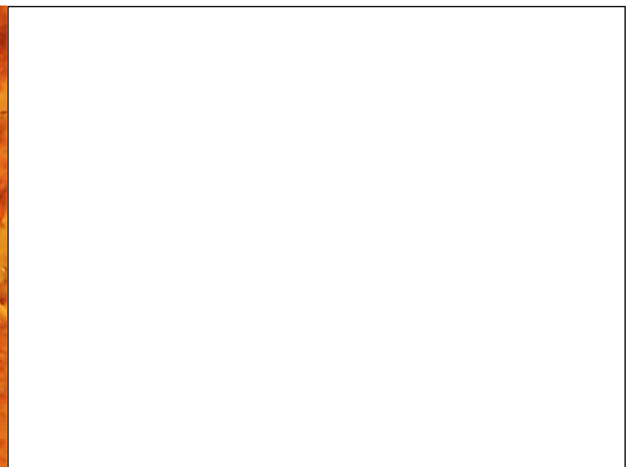


T4



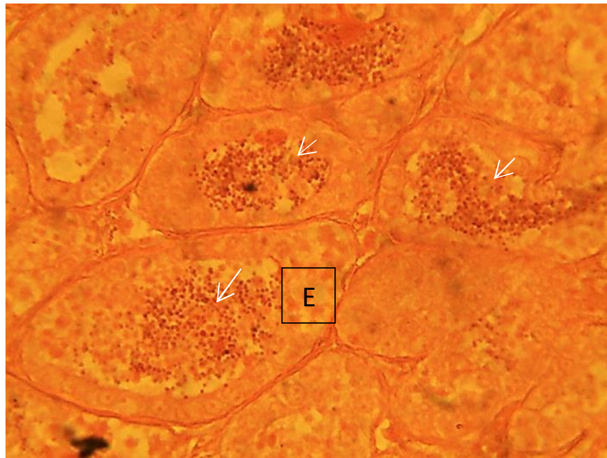
T5



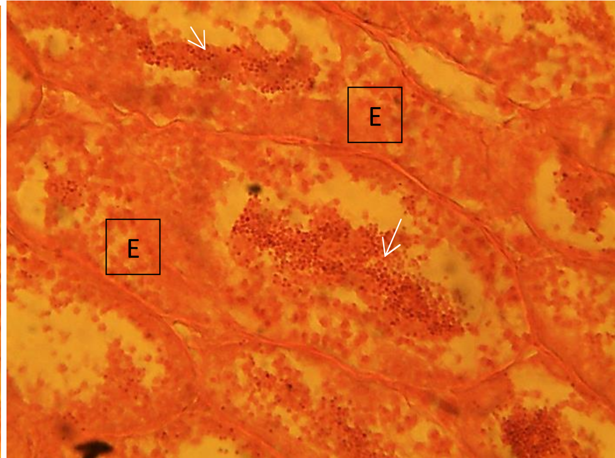


WEEK 5

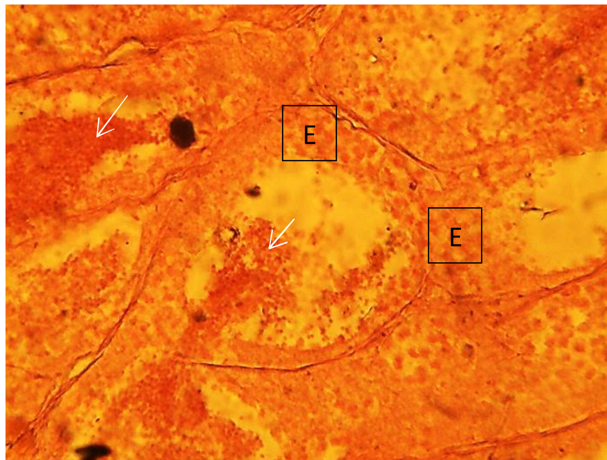
T1



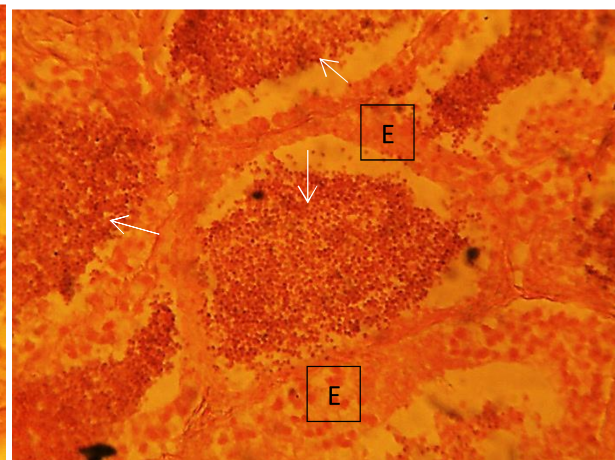
T2



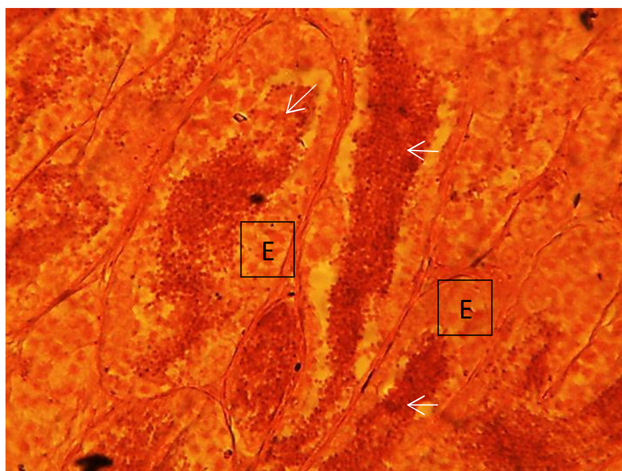
T3

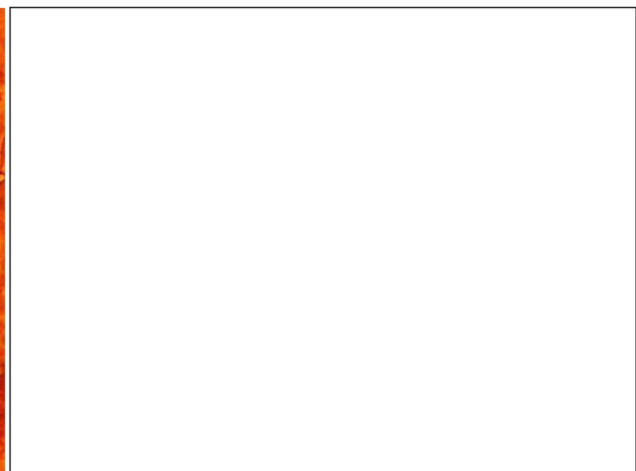


T4



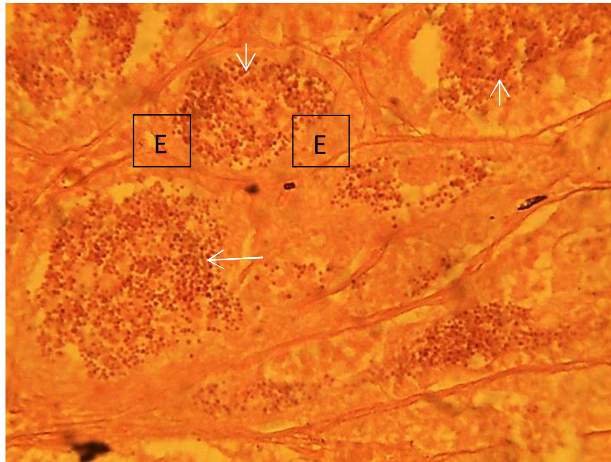
T5



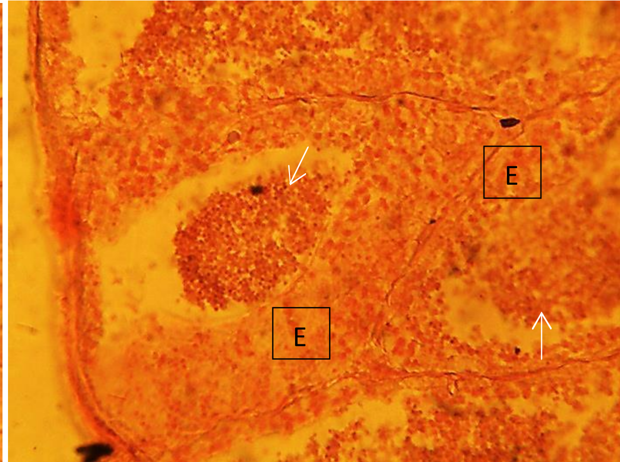


WEEK 7

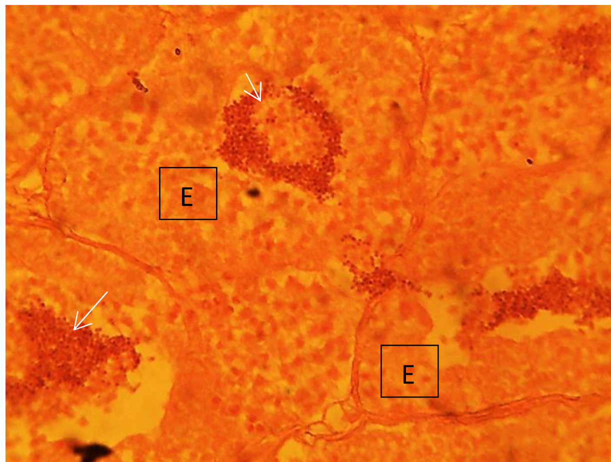
T1



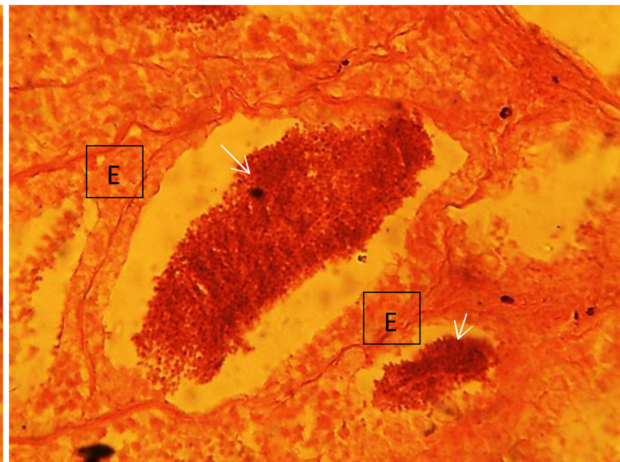
T2



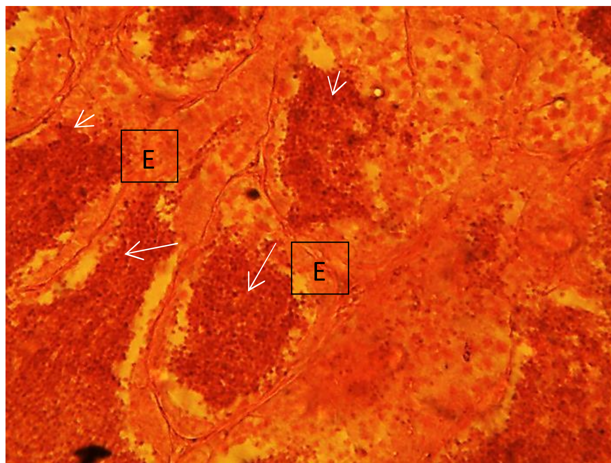
T3

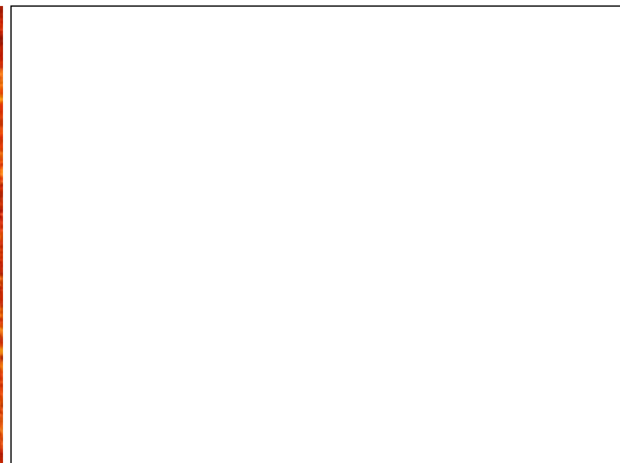


T4



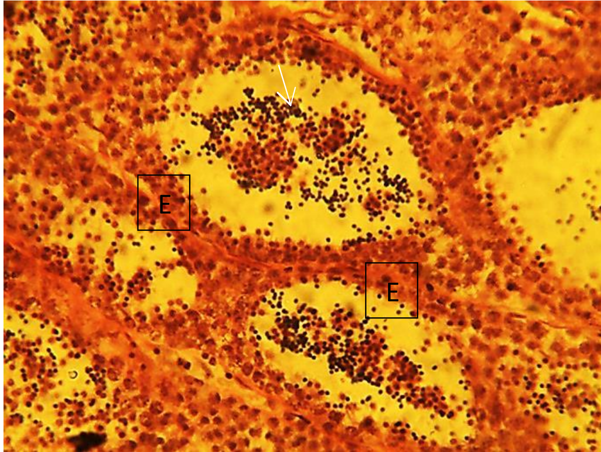
T5



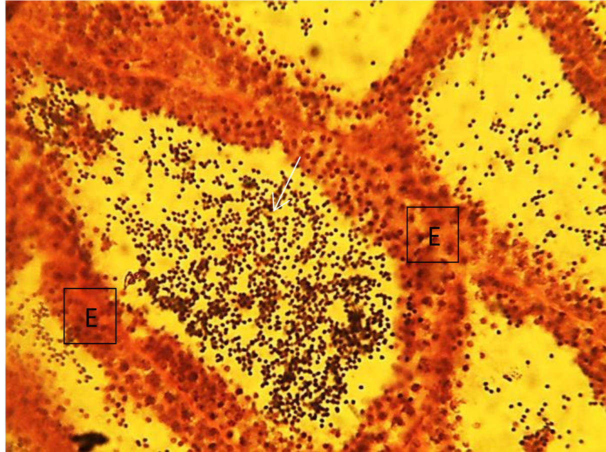


WEEK 9

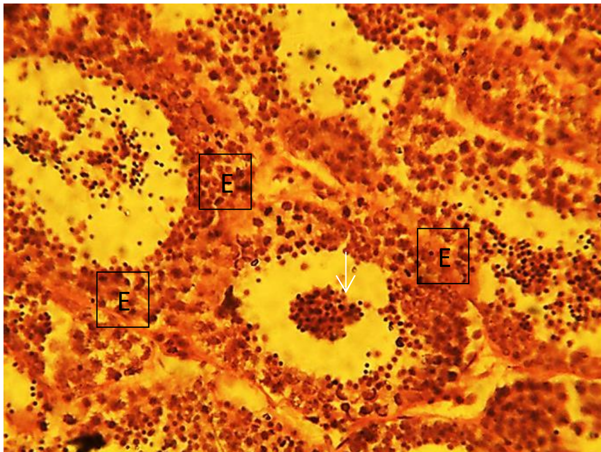
T1



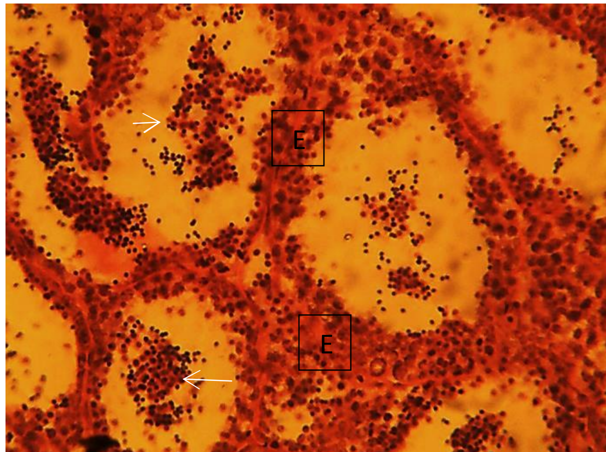
T2



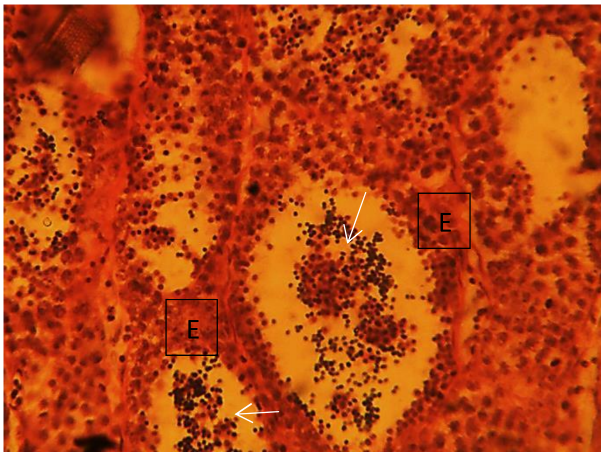
T3

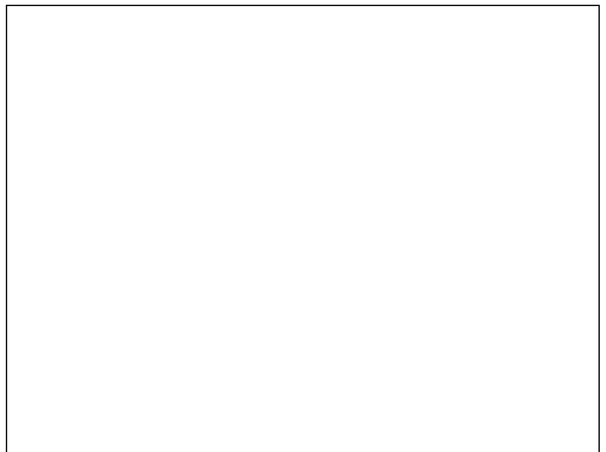


T4



T5





4.3.4 Histomorphometrical Studies

The results of histomorphometric studies of the testes of the *C. gariepinus* catfish showing the testicular diameter and the epithelial height are presented in Table 22.

Table 23: Tubular diameter and epithelial height of testes of *C. gariepinus* catfish fed diets containing varying inclusion levels of CBM.

WEEKS	TRAIT	T1 (Control)	T2 (10% CBM)	T3 (20% CBM)	T4 (40% CBM)	T5 (50% CBM)	SEM
WEEK 3	**T.D (mm)**	0.072^{a}	0.071^{a}	0.070^{ab}	0.067^{c}	0.069^{b}	0.001*
	E.H (mm)	0.0120^{b}	0.0123^{b}	0.0123^{b}	0.0133^{a}	0.013^{a}	0.000*
WEEK 5	**T.D (mm)**	0.072^{a}	0.073^{a}	0.070^{ab}	0.070^{ab}	0.069^{b}	0.001*
	E.H (mm)	0.0123^{ab}	0.013^{a}	0.0124^{ab}	0.0120^{b}	0.0120^{b}	0.000*
WEEK 7	**T.D (mm)**	0.073^{a}	0.073^{a}	0.070^{ab}	0.0703^{ab}	0.0657^{b}	0.001*
	E.H (mm)	0.0123^{ab}	0.0130^{a}	0.0120^{b}	0.0120^{b}	0.011^{c}	0.000*
WEEK 9	**T.D (mm)**	0.0731^{b}	0.0749^{a}	0.0733^{ab}	**0.066^{c}**	0.065^{c}	0.001*
	E.H (mm)	0.0127^{b}	0.014^{a}	0.0120^{b}	0.0117^{bc}	0.0113^{c}	0.000*

[abc]Means with different superscripts are significantly different at P<0.05; TD: Tubular diameter; EH: Epithelial height

Results were recorded from Hematoxylin & Eosin (H&E) stained testicular tissue sections from all the groups and their replicates used for histomorphometric evaluations. All the experimental group showed testicular histology which appear to be in the third stage of maturation (stage III) and were significantly different at the different weeks of sampling.

In all the weeks considered, the tubular diameter and epithelial height were significantly (P<0.05) different among the treatment groups. At week 3, T4 and T5 had the highest values for testicular epithelial height which differed significantly from those of T2, T3, and the control. Also, T4 and T5 showed the lowest values for tubular diameter which was significantly (P<0.05) lower than those of T2 and the control, but similar to that of T3. At week 5, the mean T.D values were significantly (P<0.05) least for T5 group whereas T1 and T2 had the highest values. Fish in T3 and T4 had testicular T.D. values which were not significantly (P×0.05) different from other groups. On the other hand, fish in T4 and T5 had significantly least mean testicular E.H. while fish in T2 group had the highest mean values. Fish in T1 and T3 had values which were not significantly (P×0.05) different from other groups. Similar results were obtained for the different groups at week 7. At week 9, fish in T4 and T5 had significantly lowest mean T.D values while those of T2 had the highest value. The control had mean T.D values which is significantly higher

than T4 and T5, lower than T2, but similar to T3. Similarly, the fish in T5 group significantly lowest testicular E.H values which differed from those of the T1, T2, and T3 fish. The T2 fish had the highest significant values which differed significantly from those of control (T1) and T3. The T4 fish had value which did not significantly differ from T3, T5 and the control group.

The presence of catechin and epicatechin in cocoa was shown to significantly increase the total antioxidant capacity of the plasma and decreases plasma thiobarbituric acid reactive substances. Flavonoids and procyanidins were found to prevent lipid oxidation through interaction between lipid forming membranes and the adsorption to the polar lipid head groups (Verstraeten *et al*., 2005). Although most studies have attributed the beneficial effect of cocoa or cocoa products on the health benefits of polyphenols (Cooper *et al*., 2008), it should be noted that cocoa and cocoa products in addition to their high content of polyphenols, are also rich in methylxanthines (theobromine and caffeine) compounds which have been reported to have negative effects on reproductive health at high dosages (Wang *et al*., 1992). Similar to the results obtained from this study, high dose of cocoa extract containing high theobromine levels was reported to greatly alter testicular structure (Maleyki and Ismail, 2008), cause testicular atrophy and aspermatogenesis in rats (Weinberger *et al*., 1978). Contrary to this results, feeding male dogs theobromine (from 25 to 150mg/kg b.w. per day) up to a year failed to cause testicular atrophy (Gans *et al*., 1980). It is not also very toxic in humans as reported by Gans *et al*. (1980). This differences in the response of the rats and dog to the high theobromine levels was attributed to the species differences in the rate of theobromine catabolism (Adamafio, 2013).

4.4 Effect of dietary inclusion of cocoa bean meal on haematology of *C. gariepinus* catfish

The haematological indices of *C. gariepinus* catfish fed different dietary inclusions of cocoa bean meal are presented in Table 23.

Table 24: haematological indices of *C. gariepinus* fed different dietary inclusions of cocoa bean meal

PARAMETER	T1 (0% CBM)	T2 (10% CBM)	T3 (20% CBM)	T4 (40% CBM)	T5 (50% CBM)	SEM
PCV (%)	29.00	30.00	30.5	31.67	33.33	0.59^{NS}
HB (g/dl)	5.3	5.2	5.6	5.33	5.90	0.12^{NS}
RBC (x 10^6/µl)	10.45	10.36	10.53	10.79	10.77	0.09^{NS}
WBC (x10^3/ µl)	9400^{b}	8850^{c}	9666^{ab}	9700^{ab}	10200^{a}	97.31*
NEUTROPHIL (%)	17.00^{b}	23.00^{ab}	24.67^{ab}	27.00^{a}	28.00^{a}	1.15*
LYMPHOCYTE (%)	80.00^{a}	76.00^{ab}	70.00^{b}	71.67^{ab}	73.67^{ab}	1.14*
MONOCYTE (%)	1.5	0.5	1.0	1.0	1.0	0.33^{NS}
EOSINOPHIL (%)	1.0	0.5	0.75	0.67	0.00	0.18^{NS}
BASOPHIL (%)	0.50	0.00	0.25	0.67	0.33	0.15^{NS}

***Means with different superscript are significantly different at P<0.05; WBC: white blood cell; HB: haemoglobin concentration; PCV: packed cell volume; RBC: red blood cell**

From the study, the fish in the different treatment groups showed no significant (P×0.05) differences in packed cell volume (PCV), haemoglobin (HB), red blood cells (RBC), monocytes, eosinophil, and basophils values. However, white blood cells (WBC), neutrophils, and lymphocytes significantly (P<0.05) differed among the treatment groups fed diets containing different levels of CBM. The results of haematological indices obtained in this study are similar to those reported by Dienye and Olumuji (2014) and Adewole and Olaleye (2014) and are within the recommended physiological ranges reported by Blaxhall and Daisley (1973) and Pietse *et al.* (1981) for the *C. gariepinus* catfish.

The WBC level was significantly (P<0.05) lowered from 9400 (in the control group) to 8850 when dietary CBM was increased to 10%. The values returned to normal as dietary CBM increased to 20% and 40% in T3 and T4 (9666 and 9700 x10^3/ µl respectively). Dietary CBM of 50% significantly increased blood WBC level to 10,200 which suggest a possible toxicity stress on the fish. The blood neutrophils was highest in T4 and T5 which were significantly (P<0.05) higher than that of the control. Neutrophil levels for animals in T2 and T3 (10% and 20%) were however not significantly different from those of the control and those of T4 and T5. In the

lymphocyte count, the fish in the control group had the highest value and was significantly different from T3 which had the least value. The values in T2, T4 and T5 however did not differ significantly from those of T1 and T3. The WBC values from the present study are similar to those reported by Adedeji and Adegbile (2011) (14692 ± 2400x10^3/**µ**l for WBC and 63.45 ± 8.62% for lymphocytes) and Ogueji and Ibrahim (2012) (16.75+0.36% for neutrophils and 83.25+0.36% for lymphocytes both in *C. gariepinus)*. A similar significant (P<0.05) increase in WBC was reported by Abrokwah *et al*. (2009) when 1ml of aqueous cocoa suspension was fed to rats daily for 48days to animals. Results similar to the increment in WBC with high CBM dietary inclusion was collaborated by the reports of Dienye and Olumuji (2014) when up to 50% *M. oleifera* leaf meal was included in the diet of *C. gariepinus* fish and by Bello *et al*. (2014) and Anene *et al*. (2014) who reported that WBC increases in fish fed diets supplemented with onion bulb and walnut (*Tetracarpidium conophorum*) leaf. Contrary to this report, Amao *et al*. (2012) reported that diets containing up to 20% inclusion of cocoa bean shells did not affect the considered haematological indices in *C. gariepinus* fish. White blood cells (leukocytes) are the defense cells of the body which yield 5 cell types (Neutrophils, eosinophil, basophil, monocytes and lymphocytes) on cell differential analysis. It was shown by Douglas and Jane (2010) that its amount has implication in immune responses and the ability of the animal to fight infection. Higher WBC count than the speciesø physiological range is usually considered to be associated with microbial infection or the circulating system, while low WBC means a decrease in the number of disease fighting cells circulating in the blood of fish (Oyawoye and Ogunkunle, 1998; Anene *et al*., 2014). The result of this study could possibly be interpreted to mean that at lower level of 10%, CBM posed a stress to the fish thereby compromising the fish immune system but as the level increased to 50%, the fish adjusted and its capacity to build immunity against pathogens was enhanced. Similarly, Abrokwah *et al*. (2009) reported that the significant increase in white blood cells meant that the administration of cocoa could boosts the immune system, since these cells are the most important cells responsible for the protection and fighting of infection. Furthermore, Bello *et al*. (2014) concluded that alkaloids and flavonoids (which are high in onion as well as cocoa) may play a role in the immune stimulation and in the function of organs (spleen, thymus and bone marrow) that is related to blood cell formation. This supports and explains an earlier observation that cocoa promotes superlative health and prevents diseases particularly viral ailments by strengthening the immune system (Addai, 2009).

4.5: Effect of dietary inclusion of cocoa bean meal on blood lipid profile of *C. gariepinus* catfish

The result of the blood lipid profile of *C. gariepinus* catfish fed different dietary levels of CBM is presented in Table 24.

Table 24: Blood lipid profile of *C. gariepinus* fed varying dietary inclusions of CBM

Indices	T1 (0% CBM)	T2 (10% CBM)	T3 (20% CBM)	T4 (40% CBM)	T5 (50% CBM)	SEM
HDL (mg/dl)	58.67	60.75	62.0	63.33	68.5	1.39[NS]
LDL (mg/dl)	49.5[a]	40.5[ab]	38.5[ab]	29.00[bc]	25.00[c]	2.63*
TC (mg/dl)	82.0[ab]	87.0[a]	89.5[a]	74.67[ab]	68.33[b]	2.78*
TGC (mg/dl)	109.00[a]	98.00[ab]	92.75[b]	104.33[ab]	96.67[ab]	2.19*

***Means with different superscript are significantly different at P<0.05; HDL: high density lipoprotein; LDL: low density lipoprotein; TC: total cholesterol; TGC: triglycerides**

From the result, there were significant (P<0.05) differences among the different treatment groups fed diets with varying inclusion levels of cocoa bean meal (CBM) in values of low density lipoprotein, total cholesterol and triglycerides. The high density lipoprotein however was not significantly (P×0.05) different among the treatment groups. The LDL values for T2 (40.5 mg/dl) fish did not differ significantly (P×0.05) from that of the control group which had the highest level (49.5 mg/dl). When dietary levels of CBM were increased to 20 and 40%, LDL levels were significantly (P<0.05) lower (38.5 mg/dl and 29.00 mg/dl respectively). Further significant (P<0.05) decrease in LDL was observed at 50 % (T5) CBM which had the least LDL level (25.00 mg/dl) and differed significantly (P<0.05) from other treatment groups except T4. At up to 40% dietary CBM level, the total cholesterol did not differ significantly (P×0.05) from the control group. However, T5 with the least total blood cholesterol differed significantly (P<0.05) from values for T3, T2, and the control group but was not significantly different from T4 and control (T1) values. TGC was significantly lower in T3 (92.75 mg/dl) than T1 (control) (109.00 mg/dl). The T3 group did not differ significantly from T2 (98.00 mg/dl), T4 (104.33 mg/dl), and T5 (96.67 mg/dl). On the other hand, T1 (control) was statistically similar to T2, T4, and T5.

This results collaborates that of Abrokwah *et al*. (2009) who observed that oral administration of 1m of aqueous cocoa suspension to rats for 48 days did not have any significant effect on HDL but significantly lowered total cholesterol, LDL, and TCG. Also similar reduction in blood lipid

values were reported by Anosike *et al*. (2014) who administered rats with 200mg and 400mg of methanolic extract of cocoa per kg body weight in combination with H_2O_2; and Jalil and Ismail (2008) who administered hyperlipidemic rats with 3% and 15% cocoa powder which contained 56 and 265mg theobromine respectively. Furthermore, Corti *et al*. (2009), reported lowered plasma levels of LDL and oxidized LDL, while the HDL levels of hypercholesteromic patients were increased on consumption of flavanol-rich cocoa. Magistrelli *et al*. (2014) reported that feeding diets containing 10% of ground cocoa husk to fattening pigs for 6 weeks increased plasma HDL which was corroborated by Araya *et al*. (2001) and Baba *et al*. (2007) who reported increased serum HDL in human and animal studies with intake of polyphenols. Although the mechanism by which the polyphenolic compounds exerted their function in improving the lipid profile is not clear (Khan *et al*., 2012), the significant dose dependent reduction in the LDL-cholesterol and increase in HDL with the increase in dietary CBM suggests a hypocholesteromic effect of cocoa through a possible modulation on LDL-cholesterol (Abrokwah *et al*., 2009; Anosike *et al*., 2014). On the other hand, results of clinical trials suggest that theobromine but not flavonoids is responsible for the increase in HDL levels in individuals taking cocoa products (Neufingerl *et al*., 2013). Flavanols in cocoa have been reported to be capable of modulating and preventing the oxidation and increase in LDL-cholesterol, which could put a subject at a higher risk of coronary heart disease (Osakebe *et al*., 2000; Pietla, 2000). This prevention of the oxidation of LDL-cholesterol is related to the mechanism of protecting the heart against heart disease. Since according to Abrokwa *et al*. (2009) low levels of blood triglycerides help prevent diseases like stroke and hypertension. The reduced blood triglycerides observed, will enhance a healthy living. Stearic acid which is a saturated fatty acid abundant in cocoa is easily converted to oleic acid ó a monounsaturated fatty acid, which thus causes no health problem (http://www.steviasweetener.org/stearicacid.html). Also, stearic acid has been reported to cause the reduction of plasma cholesterol by limiting its absorption and enhancing the excretion of endogenous cholesterol (Schneider *et al*., 2000).

CHAPTER FIVE

5.1 CONCLUSION

The results of the study has shown that inclusion of cocoa bean meal in the diet of catfish has a significant effect on growth, gonadal development, haematological indices, and blood lipid profile. There was an observed improvement in growth and gonadal development of the catfish juveniles. The T2 with 10% CBM dietary inclusion had the best results in the study. Above this level however, the measured characteristics were seen to be affected negatively which was supported by the haematological parameters. Furthermore, the blood lipid profile showed that increasing dietary levels cocoa bean meal for African catfish improved blood lipid profile in a linear progressive pattern. It was hence concluded that above 20% toxic effect of theobromine was prominent, or rather, there was an interaction between the theobromine and polyphenolic content which resulted to similar or declined performance when compared with those of the control group.

5.2 RECOMMENDATION

The beneficial results observed for T2 was attributed to the polyphenolic components of cocoa powder. Further research is needed to elicit clearly if this effects were truly due to polyphenols by feeding only polyphenols to them without other cocoa constituents. As seen in the study, feeding diets containing 10% CBM to catfish appeared to yield the best result and thus could be recommended to catfish breeders for use in their feeding activities. Hence 10% CBM dietary is recommended based on the results of the study.

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