

**SEMEN QUALITY AND FERTILIZING ABILITY OF INDIGENOUS NIGERIAN
TURKEY TOMS (*Meleagris gallopavo*) FED VARYING LEVELS OF PROTEIN
DIETS**

BY

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NIGERIA**

AUGUST, 2015

TITLE PAGE

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DIETS**

By

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NIGERIA**

AUGUST, 2015

DECLARATION

I declare that the work in this thesis titled “**Semen quality and fertilizing ability of indigenous Nigerian turkey toms (*Meleagris gallopavo*) fed varying levels of protein diets**” is original, and has been carried out by me in the Department of Theriogenology and Production, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis has been presented for another degree or diploma at this or any other Institution previously.

Muhammad Sanusi YAHAYA

Name of Student

Signature

Date

CERTIFICATION

This Thesis titled “**SEMEN QUALITY AND FERTILIZING ABILITY OF INDIGENOUS NIGERIAN TURKEY TOMS (*Meleagris gallopavo*) FED VARYING LEVELS OF PROTEIN DIETS**”, by Muhammad Sanusi YAHAYA meets the regulations governing the award of the degree of Master of Science in Theriogenology of the Ahmadu Bello University, and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This work is dedicated to my beloved, wonderful parents; Malam Yahaya Muhammad and Malama Fatimatu Hassan, for their love, prayers and guidance; and also to my wife (Dr. Saadiya Saad Mashi) and children (Fatima and Aisha), for their patience and understanding during the entire period of this work.

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ABSTRACT

Nutrition, particularly crude protein level, has been shown to enhance reproduction in both the male and female. The aim of the present study was to investigate the influence of dietary protein level on the fertility of indigenous turkey toms. Fifteen toms allotted to three groups of five and nine hens allotted to three groups of three were used in the study. The females were fed commercial layer mash containing 16% crude protein (CP), while the males were fed varying levels of protein, 12% CP, 16% CP and 20% CP for groups 1, 2 and 3, respectively. Semen samples were collected from the toms twice weekly for thirteen weeks. The samples were pooled by groups and evaluated. Ejaculate volume, semen concentration, semen pH, gross and individual motilities, live and dead sperm and sperm morphology were investigated and recorded. Data were summarized as mean $\pm$ SEM (Standard Error of the Mean). The semen samples were used to inseminate hens, whose eggs were collected to test for the fertilizing ability of the sperm cells *in vivo* on group basis. The semen was further used to perform an *in vitro* assay to test the fertilizing ability of spermatozoa on individual basis. At the end of the experiment, three toms from each group were sacrificed and the testicles, epididymis and *vas deferens* were used for analysis of sperm reserves. The mean ($\pm$ SEM) body weights of all the three groups did not differ significantly ($P > 0.05$). Groups 2 and 3 had significantly ($P < 0.05$) higher values of ejaculate volume (0.246 ± 0.015 ml and 0.250 ± 0.018 ml respectively.), semen concentration ($6.992 \pm 0.560 \times 10^9$ and $7.766 \pm 0.612 \times 10^9$ respectively), gross motility (79.09 ± 1.36 % and 80.00 ± 1.19 % respectively), individual motility (85.58 ± 1.26 % and 85.39 ± 1.12 % respectively), live sperm count (81.14 ± 1.32 % and 82.802 ± 1.12 % respectively) and lower values of total sperm defects (17.31 ± 0.996 % and 16.89 ± 0.804 % respectively), than Group 1 which had (0.165 ± 0.008 mLs), ($5.326 \pm 0.428 \times 10^9$), (73.01 ± 1.34 %), (76.81 ± 1.36 %) and (18.425 ± 1.07 %) for ejaculate volume, semen concentration, gross motility, individual motility, live sperm count

and total sperm defects, respectively. There was positive correlation between body weights and semen parameters ($P < 0.05$) in all the groups. The sperm penetration assays revealed significantly ($P < 0.05$) higher number of sperm penetration holes in Groups 2 and 3, for both *in vivo* (160.97 ± 8.084 and 172.83 ± 7.647) and *in vitro* (187.96 ± 8.121 and 189.16 ± 6.446) assays, than in Group 1, (136.80 ± 9.283) and (148.88 ± 9.159) respectively. The gonadal sperm reserve was significantly higher ($P < 0.05$) in Groups 2 ($0.206 \pm 0.003 \times 10^9$) and 3 ($0.211 \pm 0.003 \times 10^9$) than in Group 1 ($0.185 \pm 0.003 \times 10^9$). The epididymal and *vas deferens* sperm reserves, $0.175 \pm 0.002 \times 10^9$ and $3.750 \pm 0.274 \times 10^9$, were higher ($P < 0.05$) in Group 3 than in Group 2 ($0.115 \pm 0.009 \times 10^9$ and $2.817 \pm 0.221 \times 10^9$) and Group 1 ($0.079 \pm 0.003 \times 10^9$ and $2.000 \pm 0.058 \times 10^9$). It is concluded that 20% CP had the most positive influence on the semen quality and fertilizing ability of indigenous Nigerian turkey toms; followed by 16% CP, with 12% CP exerting the least positive influence. Further work is recommended to determine optimal level of crude protein, energy and other essential feed components for the indigenous Nigerian turkey tom. Farmers should adopt a dietary protein level of 16-20% CP for the local turkey toms pending the determination of the optimal level.

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LIST OF ABBREVIATIONS

AI Artificial Insemination

CP Crude Protein

DMEM Dulbecco's Modified Medium

GD Germinal Disc

Hr hour

i.e That is

IPVL Inner Perivitelline Layer

N/E Nigrosin/Eosin

NaCl Sodium Chloride

OPVL Outer Perivitelline Layer

PBS Phosphate Buffered Saline

PL Perivitelline Layer

PVM Perivitelline Membrane

SP Sperm Penetration

SST Sperm Storage Tubules

TES Tris Buffer

yr Year

PAS periodic Acid Schiff

EDS Ethane Dimethane Sulphonate

ES Ectoplasmic Specialization

LH Leutenising Hormone

FSH Follicle Stimulating Hormane

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Fertility is a complex interaction of traits involving two individuals of different genetic composition, that is, male and female, and it is their ability to mate and produce viable offspring (Foote, 2002). Fertility is a major factor in the success of any breeding programme, it depends on numerous factors. Success starts with a male animal that is healthy, disease-free, and produces ample quantity of high quality semen. However, equally important is the fertility potential of the female and also the environmental influences. Each of these components must be maintained at high standard as maximum reproductive efficiency will be dependent on the high standard of each of the components (Kirby *et al.*, 1998). Semen quality is an important component of male reproduction, and like other phenotypic expressions, it no doubt consists of a genetic component, an environmental component and a variety of interactions between the two (Foote, 1978). Nutrition has been shown to have a significant effect on semen quality traits (Sotirov *et al.*, 2002). Optimum feeding is essential to maintain males in good reproductive state (Rekwot *et al.*, 1994). Starvation on the other hand leads to a marked reduction in semen production, but this can be almost completely reversed in about 14 days after the resumption of normal feeding (Obidi *et al.*, 2008). Studies on specific nutrient requirements for semen production are rare, especially in indigenous poultry species (Obidi *et al.*, 2008). Some studies on dietary calcium levels on semen characteristics of chicken showed that semen characteristics and fertility differed insignificantly. During the growth of males, dietary protein influences the onset of sexual maturity, 9% crude protein diet

or less delays sexual maturity in chicken (Surai, 2002). Adult White Leghorn cocks fed 9% dietary protein produced normal quantities of semen, and the diet contained 1% calcium and had an energy value of 2853 Kcal/kg (Lake, 1971). Pana *et al.* (2000) showed that Cornish broiler cocks, whose daily feed consumption was limited to 130 g/cock/day produced ejaculates whose sperm concentration did not differ significantly from those of their full-fed counterpart. Ezekwe *et al.* (2003) found that the effects of underfeeding local cocks on the semen quality traits appear to be more severe on the physical rather than on the biochemical characteristics. This shows that the spermatogenic functions of the testes are more responsive to underfeeding than the secretory activity of the reproductive tract. Underfeeding diminished the ability of the gonads to respond to gonadotropin stimulation (Davies *et al.*, 1957). Moderate underfeeding or reduction of feed intake by 30% to 50% adversely affects semen production and quality (Ezekwe *et al.*, 2003).

It is necessary to evolve a feeding programme for breeding birds that will improve their reproductive efficiency, reduce the high cost of feeding and does not inflict damage to their reproductive functions. Protein level is a limiting factor in the diet of birds; its optimal level is a prerequisite not only for a rapid growth, but also for the normal condition of breeders by influencing the quantitative and qualitative parameters of semen (Sotirov *et al.*, 2002). The studies by Meyer *et al.* (1980) on the volume of ejaculate and the concentration of spermatozoa showed that the feeding of exotic male turkeys (toms) with diets containing 12 % and 17 % protein, respectively resulted in significantly better results when the high dietary protein level was used. According to previous investigators (Cherms *et al.*, 1981; Brown, 1982; Cecil, 1984; Cecil, 1986; and Sexton 1986), the low dietary protein content (12.8 %) decreases the quality of semen. The use of 17 % dietary protein levels in turkey toms up to the age

of 28 weeks and 8 % protein thereafter resulted in a satisfactory sperm production up to the end of the breeding period (age of 47-52 weeks) (Cecil, 1986).

Artificial insemination (AI) has been a critical component of reproduction in turkeys since the 1960s, and is used almost exclusively for commercial flock production in some areas of the world (Foote, 2002). The contrast in the size of the toms and hens and consequent low fertility of the males after natural mating has resulted in the complete integration of AI in commercial production. Reproductive performance is critical to efficient production in poultry and selection of males based on semen characteristics have been suggested in roosters and toms (McDaniel *et al.*, 1998). Success in AI requires quality semen which has the capacity to reach the site of fertilisation, fertilize the egg and activate embryonic development (Vanessa and Mariana, 2008).

1.2 Statement of Research Problem

Poultry production is gaining popularity in the developing countries due to its role in bridging the protein malnutrition, economic empowerment of the resource poor segment of the society. It also fits well in the farming system commonly practiced (King'ori, 2011). The continued supply of day old chicks and poults is very essential for the success of the operation (Zahraddeen *et al.*, 2005). Decreased fertility resulting from natural mating associated with the indigenous turkey toms is militating against its ability to fit well into this husbandry system. The integration of AI in the production system is a possible solution to the problem. The females have the ability to store sperm for a long time, it is therefore necessary to have males with the potentials to produce large quantities of good quality semen for successful breeding, especially AI (Sotirov *et al.*, 2002). Semen production is less effective in the Nigerian local turkey toms compared to the exotic breeds, probably due to genetic and

environmental effects (Zahraddeen *et al.*, 2005). Feeding, as an environmental factor, is essential to keep males in good reproductive condition and for the production of ample quantity of good quality semen (Rekwot *et al.*, 1994). The feed requirement for the indigenous male turkeys for optimum sperm production is an aspect that has paucity of information.

1.3 Justification

One of the most important factors in poultry breeding is the identification of males with high fertilizing ability. A number of factors apart from genetic conditions affect this ability and they include age, season, amount of light, state of health and nutrition (Paluch *et al.*, 2013). Dietary protein level in birds is a limiting factor and the optimal level is a prerequisite not only for rapid growth, but also for normal condition of breeders by influencing the quantitative and qualitative parameters of semen (Sotirov *et al.*, 2002). Adequate feed is essential to maintain males in good reproductive condition, and starvation leads to a marked reduction in semen production which can be completely reversed after resumption of normal feeding for about 14 days (Obidi *et al.*, 2008). There is paucity of information on the optimum protein requirement in the indigenous turkey toms for optimum semen production and fertility. There is need therefore to determine an optimal level of dietary protein for the indigenous Nigerian turkey toms for optimum semen production and fertility. This research is expected to provide some information on the protein requirement of the indigenous Nigerian turkey tom with regards to reproduction.

1.4 Aim of the Study

The aim of the research was to assess the effects of different dietary protein levels on the fertility of indigenous Nigerian turkey toms

1.5 Specific Objectives

The specific objectives were to:

- 1) Assess the effect of feeding turkeys (toms) with different crude protein levels on semen characteristics.
- 2) Assess the fertilizing ability of semen from toms fed varying levels of protein diets through *in vivo* and *in vitro* sperm penetration assay
- 3) Determine the effect of crude protein level on the gonadal and extragonadal sperm/spermatid reserve of turkey toms fed varying levels of protein diets.

1.6 Null Hypotheses

H₀₁; There is no difference in the semen characteristics of toms fed varying levels of protein diets that are isocaloric.

H₀₂ There is no difference in the fertilizing ability of semen from turkeys fed varying levels of protein diets that are isocaloric.

H₀₃ There is no difference in gonadal and extragonadal sperm reserves between turkeys fed varying levels of protein diets that are isocaloric.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

The turkey (*Meleagris gallapavo*) was domesticated in the New World, but its specific origins are somewhat unclear (Buss, 1989). Archaeological specimens of wild turkey have been found in North America that date to the Pleistocene, and turkeys were emblematic of many Woodland tribes in North America. The earliest signs of domestication found to date appeared in Maya sites such as Cobá beginning about 100 BC-100 AD (Beacham and Stephen, 2007).

2.2 Turkey Species

The wild turkey (*Meleagris gallopavo*) is indigenous to much of the eastern and southwestern United States (US), Northern Mexico and Southeastern Canada. Six subspecies are recognized by biologists: Eastern (*Meleagris gallopavo silvestris*), Florida (*M. g. osceola*), Rio Grande (*M.g. intermedia*), Merriam's (*M.g. merriami*), Gould's (*M.g. mexicana*), and Southern Mexican (*M.g. gallopavo*). The differences among them are primarily habitat, but there are minor differences in body size and plumage colouration (Mock, 2002). The ocellated turkey (*Griocharis ocellata* or *Meleagris ocellata*) is considerably different in size and coloration and thought by some a separate species entirely (Mock, 2002). It is native to the Yucatán Peninsula of Mexico and, interestingly enough, is today often found wandering in Maya ruins such as Tikal (Guatemala). The ocellated turkey is more resistant to domestication, but was among the turkeys kept in pens by the Aztecs as described by the Spanish (Beacham and Stephen, 2007). Turkeys were used by pre-Columbian North American societies for meat and egg, and their feathers were prized for decorative objects and clothing.

The hollow long bones of turkeys were also adapted for use as musical instruments and bone tools (Mock, 2002).

2.3 Turkey Domestication

At the time of the Spanish colonization, there were domesticated turkeys both in Mexico among the Aztecs, and in the Pueblo societies of the American southwest. Evidence suggests that the turkeys from the southwest were actually imported from Mexico about AD 300, and perhaps re-domesticated in the southwest about 1100 AD, when turkey husbandry really took off (Buss, 1989). Wild turkeys were found by the European colonists throughout the eastern woodlands. Variations in coloration were noted in the 16th century, and many turkeys were carted back to Europe.

Evidence for turkey domestication accepted by scholars which were obtained from archaeological facts includes; the presence of turkeys outside of their original habitats, the presence of constructed turkey pens; evidence of turkey burials; presence of healed long bone fractures, and the presence of quantities of the eggshell in various places away from their original habitat (Beacham and Stephen, 2007). A work using patterned calcium absorption in egg shell to pick out shell, which came from hatched eggs has added another possible route of investigation (Mock, 2002).

2.4 Artificial Insemination

Bees and many other flying insects have played an important role in plant reproduction through AI for a very long time (Foote, 2002). The use of AI technology in animals is a human invention and is more recent. There are prehistorical tales of Arabs obtaining semen from mated mares, belonging to rival groups and using the sperm to inseminate their own mares (Foote, 2002). The developments that made AI the most important animal biotechnology applied to date include improved methods

of male management and semen collection, evaluation, preservation, and insemination (Foote, 1998).

The springing up of AI was a result of several efforts by numerous researchers. Leeuwenhoek and his assistant, Hamm, first saw sperm cells, which they called “animalcules” (Leeuwenhoek, 1678). The first documented successful insemination was performed in dog by Spallanzani (1784). Pioneering efforts to establish AI as a practical procedure were begun in Russia by Ivanoff in 1907 (Foote, 2002). Ivanoff studied AI in domestic farm animals, dogs, foxes, rabbits, poultry and horses (Ivanoff, 1922). He developed semen extenders and trained technicians to select superior stallions and multiply their progeny through AI (Ivanoff, 1922). These brought enormous improvement over the earlier methods of collecting semen from sponges placed in the vagina of mount animals (Foote, 2002).

Semen collection, processing, and AI have been reviewed (Sexton, 1979; Lake, 1986; Donoghue and Wishart, 2000). Pioneers in the poultry field were Burrows and Quinn (1937), who worked in the development of abdominal massage and pressure method to collect semen. With the ease of collecting poultry semen, and proximity of hens on large breeding farms, AI became extensively useful with freshly collected semen. The technology has a fertility rate of about 100% and is exclusively used for turkey breeding because natural mating in turkeys is difficult (Foote, 2002).

2.5 Tom Selection

There is no actual breeding soundness evaluation in poultry, but it is important to select good roosters and ‘Top-Notch Toms, that is, toms whose spermatozoa have high fertilizing ability in AI of poultry (Donoghue, *et al.*, 2003). Researchers have worked to evolve and improve methods by which toms and roosters with high potency can be identified (Bakst, 1993).

Tom selection based on specific polygenic traits such as semen volume could be made on the basis of genetic markers revealed in DNA analysis or biochemical or serological analyses. Selection based on such markers could be performed soon after hatching rather than waiting for the onset of semen production (Bakst, 1993). As the technology develops, new markers linked to the production of spermatozoa, which possess a high tolerance (in terms of sustained high fertility and hatchability) to liquid storage and/or cryopreservation may be identified (Bakst, 1993).

2.6 Reproduction in Male Avian Species

2.6.1 Anatomy of the tom's reproductive tract

The primary sex organs of the birds are the testes, whose main function is the production of sperm and the male sex hormone, testosterone. Both testes are functional in the male when it attains sexual maturity. The size of the two testes may differ; the left testis is usually heavier than the right (Etches, 1996). They are located deep in the abdominal cavity and are, therefore, visible only after removal of other organs, in particular the intestine. They are suspended from the dorsal body wall in the midline adjacent to the anterior ends of the kidneys just posterior to the lungs. The testes are surrounded by a fibrous capsule that includes connective tissue and contractile fibres (Aire and Ozegbe, 2007). They contain interstitial tissue and seminiferous tubules, which are the site of spermatogenesis and, in developed testes, make up most of the testicular mass. Interstitial tissue includes Leydig or interstitial cells, the main source of testicular androgens (Mauget *et al.*, 1994; Rosenstrauch *et al.*, 1998; Madekurozwa *et al.*, 2002). In the female only the left ovary is functional, but in most male birds both testes are functional (Obidi *et al.*, 2008). In roosters the sperm production per gram of testicular parenchyma (on the average) is approximately 100×10^6 , with a daily sperm production of 2.5×10^6 spermatozoa

(Obidi *et al.*, 2008), while in turkey toms they were 131×10^6 and 560×10^6 , respectively (Cecil *et al.*, 1988). An accurate method of determining the quantity of sperm that a bird can produce is generally by measuring the circumference of the testes; for example, the larger the size of the testes, the greater the sperm production (Senger, 2003). The testes in the birds are located in the centre of the body cavity, and spermatogenesis occurs at body temperature (41°C), as opposed to the mammalian scrotal temperature of 24 to 26°C (Etches, 1996; Tuncer, *et al.*, 2006). The tom's reproductive tract comprises a duct system, with a paired epididymis and vasa deferentia. Before copulation in the birds, the *vas deferens* increases in diameter, allowing semen to be stored in the bulbous region. Semen is then released from the vasa deferentia during sexual stimulation (Perry, 1960). There are many factors that affect the seminiferous tubules. They include seasonal influences and dietary deficiencies, which may cause a reversible degeneration of the tubules and, thus, affect spermatogenesis (Anderson, 2001).

The function of the avian male is to produce semen and in the case of natural mating to copulate and ejaculate the semen into the everted cloaca of the female. As sexual maturity is attained, the weight of the paired testes increases in size (Etches, 1996). Testes in some species are of identical sizes; for example, in tree swallow (*Tachycineta bicolor*) (Kempnaers *et al.*, 2002); chickens (Hocking, 1992), but many species show testicular size asymmetry, with one testicle normally being larger in adulthood than the other (Yu, 1998; Gunn *et al.*, 2008). As in other vertebrates, the direction of testis size asymmetry within a species is generally consistent; that is, testis of a particular side is usually bigger than that of the other side, and this is fairly constant within species (Yu, 1998; Merila and Sheldon, 1999). An extreme case of avian testicular asymmetry is found in *Centropus spp.*, which develops a single testis

(Chapin, 1939; Ligon, 1997). In the turkey, however, the left testis is most often larger than the right (Lake, 1971).

It has been suggested that high ambient temperatures interfere with spermatogenesis in birds (Rekwot *et al.*, 2005), but the post-abdominal air sacs being close to the testes may reduce the testicular temperature by about 3 to 4⁰C due to air movements in inspiration and expiration (Obidi *et al.*, 2008). It has also been suggested that spermatogenesis is more intense during the night, when the activity of the birds is lowest and the body temperature reduced (Obidi *et al.*, 2008). The blood supply and the arrangement of blood vessels within the avian testes are simpler than in mammals; there is no pampiniform plexus of blood vessels as seen in mammals. The testicular artery is extremely short and branches inside the testes to form many arterial loops amongst the seminiferous tubules (Lake, 1971). Venous drainage runs to superficial veins, which unite and run directly into the posterior *vena cava*, and the common vein is short (Lake, 1971). Interstitial tissue containing blood vessels and Leydig cells, which secrete androgens is not readily visible in histological sections of the testes of birds because of the great expansion of the tubules (Deviche *et al.*, 2011). Semen is stored in the *ducti deferentes*, which end in two papillae projecting into the cloaca (vent) in the fowl, guinea fowl and turkey (Lake, 1971). The *ducti deferentes* in the duck and goose open at the base of the penis in the cloacae and semen, which is exuded from them, the semen flows down the grooves spirally arranged down the length of the projecting penis (phallus) (Lake, 1971; Deviche *et al.*, 2011) (Figure 2.1).

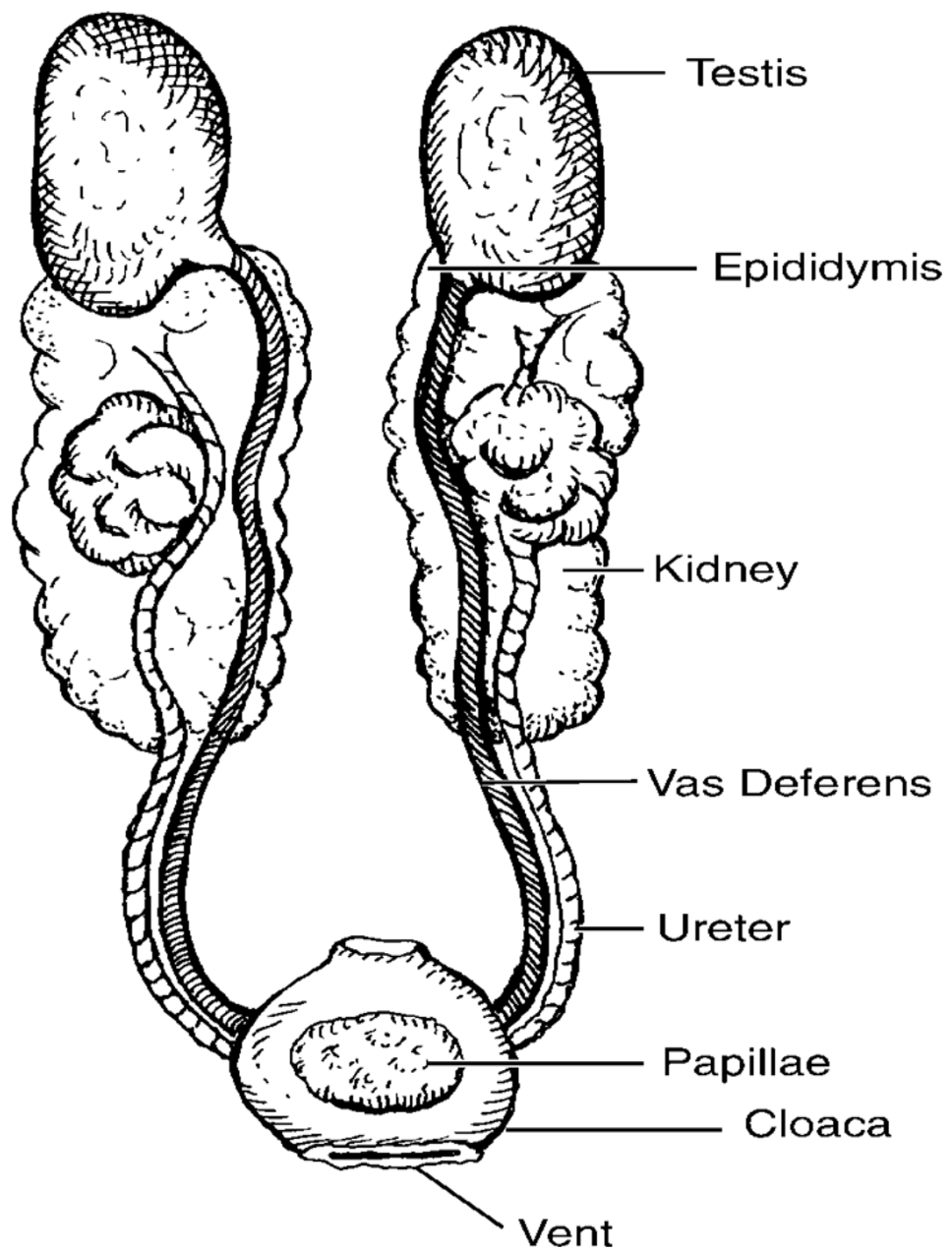


Figure 2.1: The reproductive system of the turkey tom
Source: Banerjee, (2011)

2.6.2 Physiology of turkey reproduction

Sperm production is initiated by adequate secretion of Gonadotrophin Releasing Hormone (GnRH), from the hypothalamus, the secretion of luteinising hormone (LH) and follicle-stimulating hormone (FSH) by the anterior pituitary and the secretion of the gonadal steroids (testosterone and estrogen) from Leydig cells. LH acts on the Leydig cells within the testes to stimulate the production of progesterone, which is converted to the male sex hormone, testosterone. Testosterone within the seminiferous tubules is essential for spermatogenesis, while the Leydig cells become unresponsive sustaining high levels of LH (Senger, 2003). The testes are surrounded by a layer of connective tissue, containing the seminiferous tubules and Leydig cells. Several androgens are produced in the interstitial cells of the testes, but the major hormone in the blood, is testosterone. Testosterone is essential for the development of the secondary sex characteristics and for normal mating behaviour in the males. It is also necessary for sperm production and the maintenance of the male duct system. Testosterone, which aids in spermatocytogenesis, also facilitates the transport of sperm within the male system and deposition of sperm in the female reproductive tract (Bearden *et al.*, 2004).

2.6.3 Testosterone and spermiogenesis

The role of testosterone in spermiogenesis has been studied in the testosterone-suppressed rat model by determining the Hourly Production Rate (HPR) of round spermatids during testosterone withdrawal and replacement (O'Donnell *et al.* 1994). The conversion of steps 1 to 7 round spermatids was maintained in the presence of low testosterone, but the conversion of step 7 to 8 spermatids proceeded at only 15% of its normal efficiency. Furthermore, adhesion of round spermatids at this stage appeared to be lost, resulting in their sloughing into the lumen and appearance in the

epididymis (O'Donnell *et al.* 199). When testosterone levels were restored with a high dose of testosterone, the conversion of step 7 to 8 spermatids was normalized within 4 days. The basis of the testosterone dependency of spermatid/Sertoli cell adhesion may involve effects on the Sertoli cell cytoskeleton. Normally a junctional area, termed the ectoplasmic specialization (ES), develops between Sertoli cells and round spermatids at the onset of the elongation process. The ES (or associated elements) are probably involved in the attachment of round spermatids. In the absence of testosterone, either *in vivo* or *in vitro*, this junction is disrupted (Cameron and Muffly, 1991; Muffly *et al.* 1994). Consequently, testosterone withdrawal may lead to loss of spermatid adhesion, thereby precluding their further maturation. In a recent study, the testosterone deficiency which followed the destruction of Leydig cells by (EDS) in pubertal rats resulted in the disruption of the ES and sloughing of round spermatids (Cameron *et al.* 1993). Based on Sertoli cell–spermatid co-culture experiments, the authors proposed that FSH is responsible for the alignment of the actin and vinculin filaments of the Sertoli cell cytoskeleton, which are needed for this testosterone-dependent adhesion (Muffly *et al.* 1994).

Further studies are required to establish the testosterone-dependent processes involved, especially the involvement of cell adhesion proteins known to exist within the seminiferous epithelium, such as members of the integrin and cadherin families. Both mRNA and immunostaining for N-cadherin have been found in spermatocytes and round spermatids (MacCalman *et al.*, 1993), and recently $\alpha 6\beta 1$ integrin was shown to be localized to the region of elongating spermatid heads (Salanova *et al.*, 1995). The regulation of these cell adhesion proteins during manipulation of testosterone levels *in vivo* has not been reported (McLachlan *et al.*, 1996).

As the bird reaches maturity, the production of testosterone is stimulated by the increasing concentration of circulating gonadotrophins (Etches, 1996). The major gonadotrophins involved are FSH and LH, which are both secreted by the anterior pituitary (Salisbury *et al.*, 1978). FSH acts on the germinal cells in the seminiferous tubules of the testes and supports spermatogenesis to the secondary spermatocytes stage. LH stimulates the Leydig cells to produce testosterone and other androgens (Hafez and Hafez, 2000).

2.6.4 Accessory sex glands in the avian

The prostate, vesicular and bulbo-urethral glands are the accessory glands present in most domestic animals, with their main function being the production of secretions; The secretion provide nutrition, aid in sperm transport and contain specific chemical agents (Morrell, 2011). The epididymis and the *vas deferens* are the only accessory organs present in poultry (Hafez and Hafez, 2000). The avian has no accessory sex glands. Therefore, the seminal fluid is derived entirely from the testes and the underlying ducts. Additionally when semen is collected from the toms and cockerel by abdominal massage, there are lymphatic exudates from the phallic folds, which contribute to the ejaculate (Hafez and Hafez, 2000). It is not clear, if this lymphatic fluid is a normal avian semen component, as the composition of semen during natural mating in birds has received little attention (Etches, 1996).

2.6.5 Spermatogenesis

Spermatogenesis is the process of division and differentiation by which sperm cells are produced in the seminiferous tubules of the testes. It consists of two phases namely: spermatocytogenesis and spermiogenesis (Gordon, 2005) or three phases: spermatocytogenesis, spermatidogenesis and spermiogenesis (Deviche *et al.*, 2011).

2.6.5.1 Spermatocytogenesis

Cross sections of the seminiferous epithelium form well-defined cellular associations that undergo cyclic changes. The numbers of distinct cellular stages differ in different types of domestic animals; for example, 14 distinct stages are identifiable in some species, whereas only six stages are identified in the human and 12 in the bull (Hess and de Franca, 2008). However, the duration of spermatogenesis is uniform within a species, and is estimated to be 12.8 days in the cockerel (Hafez and Hafez, 2000) and is similar in turkey toms (Noirault *et al.*, 2006). The seminiferous tubules in poultry are arranged as a network of interconnected ducts that empty into the rete testis and their periphery is lined with spermatogonia, which are normally considered to be involved in the first stage of spermatogenesis (Etches, 1996). There are two types of the cells in the seminiferous tubules; namely the germ cells or spermatogonia and Sertoli cells, which are somatic cells. Sertoli cells extend from the basement to the lumen and play a role in supporting spermatogenesis (Bearden *et al.*, 2004). The spermatogonia are specialized diploid cells found in the basal compartment of the seminiferous epithelium and are produced continuously by mitotic divisions to yield subsequent generations of spermatogonia and spermatocytes, which enter the first meiotic division. Differentiation of spermatogonia into haploid spermatocytes requires the participation of the Sertoli cells to support sperm production, which also provide the micro-environment in which differentiation can take place, and also nourishes the developing sperm (Tuncer *et al.*, 2008).

2.6.5.2 Spermiogenesis

The transformation of sphericacal, haploid spermatids into elongated highly condensed and mature spermatozoa that are released into the seminiferous tubule lumen is called spermiogenesis. This differentiation of spermatids proceeds through at

least four prolonged phases: Golgi, capping, acrosomal, and maturation phases (Ogawa *et al.*, 2005; Gordon, 2005). The phases, accomplished through about 16 steps, are useful in identifying specific stages in the cycle of seminiferous epithelium (Hess and de Franca, 2008).

Golgi: Golgi apparatus is very important during the early steps of spermiogenesis, as the formation of acrosome depends upon the ability of this organelle to produce vesicles and granules which contain the enzymatic components of the acrosomic system that covers the sperm nucleus (Neves *et al.*, 2002; Senger, 2003). Differentiation of the first three steps of round spermatid formation involves a prominent Golgi apparatus that is identified by PAS staining (Ventela *et al.*, 2002). Step 1 spermatids have a small, perinuclear Golgi region without acrosomic vesicles or granules (Oatley *et al.*, 2006). Subsequent steps 2-3 show proacrosomal vesicles and granules within the Golgi apparatus, with the formation of a single large acrosomal granule within a larger vesicle that indents on the nucleus.

Capping: This involves steps 4-5 round spermatid, with the acrosomic granules touching the nuclear envelop and the vesicle beginning to flatten into a small cap over the nuclear surface (Hess and Franca, 2005). In steps 6-7 the acrosomic vesicle becomes very thin and the granules flatten. Step 8 is the last round spermatid, and the acrosome flattens over approximately 1/3 of the nuclear surface. Step 8 nuclei begin to change shape (Neves *et al.*, 2002).

Acrosomal: Acrosomal steps 9-14 involve migration of the acrosomal system over the ventral surface of the elongating spermatid nucleus. The migration of the acrosome is by around step 14 spermatid and is difficult to identify in typical histological sections (Nakagawa *et al.*, 2007).

Maturation: In this stage there are fewer changes in nuclear shape and acrosomal migration (Hess and de Franca, 2008). The nucleus continues to condense and the acrosome matures into a thin PAS+ structure that protrudes at the apex but covers nearly all the nucleus, except for the portion connect to the tail (Nakagawa *et al.*, 2007). There is removal of excess cytoplasm which results in the formation of prominent cytoplasmic lobes and residual bodies, which contains unused mitochondria, ribosomes, lipids, vesicles and other cytoplasmic components (Nakagawa *et al.*, 2007).

2.6.5.3 Seminiferous epithelial cycle

Spermatozoa are produced within the seminiferous tubule of the testis by a process known as spermatogenesis. The process however is dependent on a sequence of activities. The various generations of germ cells in the testes of mammals and other vertebrates are not associated at random with a group of Sertoli cells, they form cellular associations which are constant (Setchell, 1982). Ortavant (1954) arranged these cellular associations or stages of the seminiferous epithelium, into an order and correctly predicted that the 4-5 generations of germ cells which are associated with a Sertoli cell sequentially develop through the stages. The period required to pass through all stages is considered to be one cycle of the seminiferous epithelium. The number of cycles which occurs from an initial division of a stem spermatogonium to the release of its daughter spermatozoa into the lumen of the seminiferous tubule is determined by the number of generations of germ cells associated with a Sertoli cell which is usually 4-5 (Ortavant, 1954). It is important to recognize the stages and their arrangement as that provides a basis for studying how spermatogenesis is regulated and for the estimation of the duration of spermatogenesis and the daily rate of sperm production of an animal (Hess and Franca 2008). However, there is no certainty that

the accepted understanding of the process of spermatogenesis described above can be applied to birds. Although Clermont (1958) and Aire *et al.*, (1980) identified eight stages in the drake and guinea-fowl, respectively. However they were able to identify a large number of 'atypical' associations. Further, Yamamoto *et al.* (1967) concluded that the cellular associations are quite variable in the Japanese quail and described cellular associations (involving up to three generations of spermatids in one association), which are fundamentally different from the pattern in mammals.

The major problem in determining the stages of the seminiferous epithelium in birds is the existence of a number of stages in one cross-section of a seminiferous tubule; for example. 6-10 in the Japanese quail (Clermont, 1958; Yamamoto *et al.*, 1967; Aire *et al.*, 1980) and overlapping of germ cells in adjacent stages makes it difficult to resolve which generations of germ cells are in one association. Moreover, the various steps of spermatid development in birds are not as easily recognized in paraffin wax sections as they are in eutherian mammals. However Lin *et al.* (1990) have solved these problems in a study they reported by examining thin sections of isolated tubules embedded in epoxy resin to resolve the structure of the developing acrosome in spermatids (Gunawardana, 1977), and by carrying out preliminary studies using a preparatory method for fixation which separated adjacent cellular associations. The report describes the stages of the seminiferous epithelium and estimates the duration of a cycle of the seminiferous epithelium of the Japanese quail. The cycle in Japanese quails is 2.69 ± 0.08 days (Lin *et al.*, 1990).

2.7 Factors Affecting Reproductive Performance

2.7.1 Nutrition

Nutrition has been shown to have a significant effect on semen quality. Optimum feeding is essential to maintain males in good reproductive state. Starvation on the

other hand leads to a marked reduction in semen production, but this can be almost completely reversed in about 14 days after the resumption of normal feeding (Boone and Hughes, 1969). Studies on specific nutrient requirements for semen production are rare, especially in indigenous poultry species (Obidi *et al.*, 2008). Ezekwe *et al.*, (2003) found that the effects of underfeeding local cocks on the semen quality traits appear to be more severe on the physical rather than on the biochemical characteristics. This shows that the spermatogenic functions of the testes are more responsive to underfeeding than the secretory activity of the reproductive tract. Underfeeding diminished the ability of the gonads to respond to gonadotropin stimulation (Davies *et al.*, 1957). Moderate underfeeding or reduction of feed intake by 30 to 50% adversely affected semen production and quality (Ezekwe *et al.*, 2003). It is considered necessary to evolve a feeding programme for breeding birds that will reduce the high cost of feeding under the intensive production system without inflicting serious damage to their reproductive functions (King' Ori, 2011).

2.7.1.1 Energy

Energy intake is one of the most important nutritional factors affecting reproduction in animals. In cattle, for example, inadequate energy intake in heifers and early lactation cows reduces reproductive performance. Excessive energy intake during late lactation and the dry period on the other hand can cause “fat cow” problems which lower reproductive efficiency in the next lactation (Funston, 2005). When poultry are fed inadequate amounts of energy, they reach sexual maturity later and drakes fed the high level of metabolizable energy (ME) or crude protein (CP) had significantly higher ejaculate volume (Ghonim *et al.*, 2010). If energy deficient rations are fed to heifers that have begun to have normal estrous cycles, they may stop cycling. An example is heifers fed diets composed mainly of poor quality hay (Ferguson, 2001).

They often will not show signs of estrus. If grain is provided, or they are put on good pasture, normal estrous cycle activity will resume as they begin to consume adequate amounts of energy (Ferguson, 2001).

2.7.1.2 Protein

Adequate amounts of protein must be provided. Prolonged inadequate protein intake has been reported to reduce reproductive performance. More recently it was found that reproductive performance may be impaired if protein is fed in amounts that greatly exceed the animal's requirements (Funston, 2005). The relationship between excess protein and reproduction needs to be clarified by additional research. During the growth of males, dietary protein influences the onset of sexual maturity. It is delayed by 9% crude protein diet or less (Surai, 2002). In studies conducted on adult White Leghorn cocks fed 9% protein, containing 1% calcium and 2853 kcal/kg energy normal qualities of semen were produced (Lake, 1971). Pana *et al.* (2000) showed that Cornish broiler cocks, whose daily feed consumption was limited to 130 g/cock/day produced ejaculates, whose sperm concentration did not differ significantly from those of their full-fed counterparts. Protein level is a limiting factor in the diet of birds; its optimal level is a prerequisite not only for a rapid growth, but also for the normal condition of breeders by influencing the quantitative and qualitative parameters of semen (Sotirov *et al.*, 2002). The study by Meyer *et al.* (1980) on the volume of ejaculate and the concentration of spermatozoa showed that the feeding of exotic male turkeys (toms) with diets containing 12 % and 17 % protein resulted in significantly better semen qualities when the (higher) 17 % dietary protein level was used. According to some investigators (Brown, 1982; Cecil, 1984; Cecil, 1986; Cherms *et al.*, 1981; Sexton, 1986), the low dietary protein content (12.8 %) decreases the quality of semen. The use of 17 % dietary protein levels up to the age of

28 weeks and 8 % protein thereafter resulted in a satisfactory sperm production up to the end of the breeding period (age of 47-52 weeks). The quality of semen was further influenced by other factors related directly or indirectly to the level and quality of feeding. It has been reported that higher reproductive performance in lower than in higher CP levels in cockerels and rams (Perry, 1960; Etches, 1996 and Jibril *et al.*, 2011). Better performance in animals fed higher CP levels than those fed lower CP levels has been shown in bulls (Rekwot *et al.*, 1987; Rekwot *et al.*, 1988), boars (Louis *et al.*, 1994), rabbit bucks (Ladokun *et al.*, 2006) and drakes (Ghonim *et al.*, 2010).

2.7.1.3 Fat

Fertility is always a major concern for turkey breeders. Considerable evidence has shown that reduced amounts of spermatozoan Poly Unsaturated Fatty Acid (PUFA) impair sperm numbers, motility, and fertilizing ability in birds (Ansah and Bucklandan, 1982; Kelso *et al.*, 1996) and mammals (Nissen and Kreysel, 1983). The previous studies may implicate spermatozoan PUFA as having an important role in sperm functionality and in optimizing fertility. Indeed, mammalian spermatozoa lipid composition has been shown to play a major role in the physiochemical modifications leading to fertilisation (Langlais and Roberts, 1985). Fat is, therefore, an essential ingredient in reproduction is concerned (Ansah and Bucklandan, 1982).

2.7.1.4 Minerals

Mineral deficiencies and imbalances are often causes of poor reproduction (Lopez *et al.*, 2004). It is clear that adequate amounts of minerals must be provided, but little is known about the effects of marginal deficiencies and imbalances. The same is true of excessive intakes of minerals which may indeed be harmful.

Phosphorus: This mineral has been most commonly associated with decreased reproductive performance in dairy cows. Inactive ovaries, delayed sexual maturity and low conception rates have been reported when phosphorus intakes are low (Lopez *et al.*, 2004). In a field study, in cattle, when heifers received only 70-80% of their phosphorus requirements and serum phosphorus levels were low, fertility was impaired (3.7 services per conception), with phosphorus supplementation however, services per conception were reduced to 1.3. Other studies indicate that the National Research Council (NRC) recommendations are adequate to maintain growth and reproductive function in heifers and cows (Funston, 2005).

Calcium: Most experimental work relating calcium to reproduction has centered on the effect of the calcium-phosphorus ratio. Controlled experiments demonstrated no effect of altered ratios on reproduction in heifers or lactating cows. Ratios (Ca:P) between 1.5:1 and 2.5:1 for lactating cows should not result in problems. However, it is the amount (grams or ounces) of calcium and phosphorus that is fed and consumed by the cow that is important. Milking cows should always be provided adequate amounts of calcium to maximize production and minimize health problems.

Some studies on dietary calcium levels on semen characteristics of chicken showed that semen characteristics and fertility differed insignificantly (Surai, 2002). A major concern in the mineral feeding of dry cows relates to providing optimum levels of calcium and phosphorus in order to decrease the occurrence of milk fever. In a study, cows treated for milk fever were 4.2 times more likely to require assistance at calving, 2 times more likely to have retained placenta and 1.6 times more likely to be treated for metritis. Since all of these disorders are associated with poorer reproductive performance, prevention of milk fever is an important consideration in maximizing reproductive efficiency (Funston, 2005).

In the domestic hen, the shell gland extracts 2-2.5 g of calcium from the blood and transfers the element, without accumulation, to the egg over a period of 15 hours (Eastin and Spaziani, 1978). Intestinal calcium uptake plays an important role in providing the amount of calcium required to perform this task (Al-Batshan *et al.*, 1994). Estrogens have a complex relationship with calcium metabolism and have been shown to increase serum calcium by increasing the density of parathyroid hormone (PTH) receptors in kidneys, and indirectly increasing the renal activity of 1-2-hydroxylase (Castillo *et al.*, 1977). The 1-2-hydroxylase is responsible for formation of 1,25-dihydroxycholecalciferol [1,25-(OH)₂ D₃] (Martz *et al.*, 1985), which is able to mediate intestinal absorption of calcium and phosphorus (Bar *et al.*, 1978) by a saturable trans-cellular route and increase the concentration of calcium binding protein in the target cells. As the hen ages, the number of eggs produced declines along with a reduction in eggshell quality, estrogen levels, and estrogen receptor population (Hansen *et al.*, 2003).

Iodine: Reproduction is influenced through iodine's action on the thyroid gland. Inadequate thyroid function reduces conception rate and ovarian activity. Thus, iodine deficiency impairs reproduction, and iodine supplementation is highly recommended when necessary (Surai *et al.*, 2004; Bearden *et al.*, 2004). Excessive iodine intakes on the other have been associated with various health problems, including abortion and decreased resistance to infection and disease in cattle (Funston, 2005).

Potassium: Some research in ruminants suggests that feeding high levels of potassium may delay the onset of puberty, delay ovulation, impair *corpus luteum* development and increase the incidence of *anaestrus* in heifers. The ration fed in this study contained approximately 5% potassium on a dry matter basis. It was fed for one year (Funston, 2005). Other studies report lower fertility in cows fed high levels of

potassium or diets in which the potassium-sodium ratio was too wide (Bearden *et al.*, 2004).

2.7.1.5 Vitamins

The vitamin requirements of ruminants are met by a combination of rumen and tissue synthesis, natural feeds and feed supplementation. Most commercial concentrates contain supplemental vitamins so the probability of infertility due to a vitamin deficiency is greatly reduced (Bearden *et al.*, 2004).

Vitamin A: is required for maintaining healthy tissue in the reproductive tract. In deficient cattle, delayed sexual maturity, abortion, the birth of dead or weak calves, retained placenta and metritis have been reported. Other problems include: delayed first estrus after calving, delayed ovulation, increased incidence of cystic ovaries, high early embryonic death and abortion and delayed uterine involution (Funston, 2005).

Vitamin E and Selenium: The deficiency of this mineral in cattle has been reported to cause retained placenta. Selenium deficiency also has been related to abortions, a high incidence of embryonic-fetal loss, poor fertility, increased incidence of metritis (Funston, 2005).

In poultry vitamin E was discovered as a “vitamin of reproduction” in 1922. In recent years it has been shown that vitamin E abounds in spermatozoa and provides antioxidant protection, especially in stress-conditions of *in vitro* semen manipulation, including dilution, storage and deep freezing of spermatozoa (Surai, 2002). Furthermore, it was shown that vitamin E provides additional protection in the case of fatty acid manipulation of the semen (Surai *et al.*, 2000; Zinini *et al.*, 2003; Cerolini *et al.*, 2005). However, the vitamin E dose-response in cockerels is non-linear (Lin *et al.*, 2005).

Selenium is an integral part of various selenoproteins with glutathione peroxidase (GSH-Px) being the first selenoprotein identified in 1973 (Surai, 2002). Selenium's participation in antioxidant defence and in the regulation of redox status of the cell could explain its importance to various physiological functions, especially to reproduction (Surai *et al.*, 1998). Chicken embryo tissues and spermatozoa are rich in long chain PUFA and as a result are very vulnerable to lipid peroxidation (Lin *et al.*, 2005). Therefore, selenium is a crucial factor in maintaining appropriate antioxidant defence during embryonic development and spermatogenesis (Surai *et al.*, 1999; Speake *et al.*, 1998). In conditions of stress in commercial poultry production, the beneficial effects of selenium are difficult to overestimate. From egg storage before incubation, when lipid peroxidation can occur, to the final day of egg production, selenium participates in preventing the damaging effects of free radicals and toxic products of their metabolism (Zanini *et al.*, 2003; Surai *et al.* 2004). Dietary antioxidant supplementation in general and organic selenium supplementation in particular are considered to be major protective measures for maintaining high productive and reproductive characteristics in poultry (Surai and Speake, 1999; Surai *et al.*, 2002).

Edens (2002) showed that, when cockerels were fed on a basal diet containing 0.28 ppm Se without additional dietary supplementation of this trace element, the percentage of normal spermatozoa was only 57.9% and two major abnormalities seen were bent midpiece (18.7%) and corkscrew head (15.4%). When this diet was supplemented with an additional 0.2 ppm Se in the form of selenite, the percentage of normal spermatozoa increased to 89.4% and abnormalities in the form of bent midpiece and corkscrew head were decreased down to 6.2 and 1.8% respectively (Edens, 2002). However, when organic selenium was included in the cockerel's diet

in the same amount, semen quality was further improved and those abnormalities decreased down to 0.7 and 0.2% and the percentage of normal spermatozoa increased up to 98.7%. These results clearly showed that the form of dietary selenium supplementation is a crucial factor of its efficiency, with organic selenium being much more effective in comparison to selenite (Agate *et al.*, 2000). Therefore selenium deficiency is associated with mid-piece damage to spermatozoa. It is clear that the mid-piece of spermatozoa of the selenium-deficient male is broken. In such conditions, sperm motility and fertilizing capacity would be compromised. Organic selenium can also improve fertility and, more importantly, increase the duration of fertility (Agate *et al.*, 2000). In particular GSH-Px activity in the utero-vaginal junction is 12-fold higher than in the liver (Papazyan *et al.*, 2006).

2.7.2 Age and breed

Age and breed of animals contribute to difference in semen production capability and semen quality. The production of semen differs according to age; very young animals that are not fully matured and very old ones have been found to perform less than the fully matured not too old ones in various animal species; for example, in goats (Butswat and Zaharadden, 1998) and cattle (Wolfe *et al.*, 2000; Kidd *et al.*, 2001)

Semen production and quality are further influenced by animal breed, in almost all animal species, no two different breeds are identical in terms of their reproduction. Breed is, therefore, an important factor affecting reproductive traits in animals. For example, in cattle Rekwot *et al.* (1988) reported difference between two breeds in terms of their semen production capacity under the same environmental conditions. In other animals, works have been done to show these differences; in cocks (Molekwa *et al.*, 2009), goat (Butswat and Zaharaddeen, 1998), guinea fowl (Butswat *et al.*, 2001) and turkeys (Zaharaddeen *et al.*, 2005).

2.7.3 Season

2.7.3.1 Ambient Temperature

Direct climatic factors acting on birds include high ambient temperature and relative humidity, resulting in severe heat stress. Heat stress is one of the main limitations in poultry production and reproduction, more especially in hot areas (Oguntunji *et al.*, 2008). Elevated environmental temperatures pose a threat to the general well-being of the birds. The increase in the body temperature without a rapid compensation of heat loss, resulting from a prolonged exposure to environmental temperature, may cause a change in the body temperature of the bird, leading to a significant impairment of semen production and reproduction (Karaca *et al.*, 2002). The intensity and duration of heat stress combined with relative humidity may also affect the behavioral, hormonal and physiological functions of birds. Such detrimental effects limit reproductive performance of the males thus inhibiting spermatogenesis and causing decrease in gonadotrophins secretion (Bah *et al.*, 2001; Rekwot *et al.*, 2005; Obidi *et al.*, 2008; Oguntunji *et al.*, 2008).

Sperm metabolism, sperm motility and sperm quality are generally lower in heat-stressed birds. Although there is paucity of research concerning the effect of hyperthermia on semen characteristics, several researchers have found that sperm can function at normal body temperature (Karaca *et al.*, 2002). Froman and Feltmann (2005) found sperm to be motile at a body temperature of 41°C, and decline with time after ejaculation.

2.7.3.2 Photoperiod or daylight length

Most domestic birds are seasonal breeders and in most birds, photoperiod stimulates spermatogenesis (Peters *et al.*, 2008). The duration and intensity of photoperiod may have an effect on the conditioning of birds for reproduction. Short days do not

stimulate gonadotrophin secretion, as they do not illuminate the photosensitive phase of the brain. However, long days illuminate the photosensitive phase and therefore the gonadotrophin LH is secreted adequately (Anderson, 2001). Time of the day for the collection of semen found to affect the quality and quantity of semen in roosters (Etches, 1996). Generally semen production is higher in the morning and in the afternoon, when it is colder (Peters *et al.*, 2008).

According to Hafez and Hafez (2000) the onset of reproduction occurs when light, acting through photoreceptors in the brain, provides neural signals which the bird's reproductive endocrine system perceives as a change in daylight length, sufficient to initiate reproduction (Hafez and Hafez, 2000).

2.7.4 Body weight

Body weight of toms and roosters is important when selecting males for breeding and flock performance. A significant negative correlation has been established between body weight and the reproductive performance in males (Harris *et al.*, 1980; Lukaszewicz and Kruszynski, 2003). It is crucial for the males to reach a minimum body weight typical for a given breed, strain or type, before they are used for breeding (Lisowski and Bednarczyk, 2005). The number of sperm per ejaculate and body weight has been positively correlated and body weight and length of the shank, comb and wattle are good predictors of semen attributes in toms and roosters as the case may be (Lisowski and Bednarczyk, 2005). This is contrary to the reported negative effect of body weight on semen production (Wilson *et al.*, 1979; Galal, 2007). Harris *et al.* (1980), however, recorded a positive correlation between body weight and semen volume in cockerels.

2.8 Semen Collection

Burrows and Quinn (1937) described a non-invasive method, the “abdominal massage method”, for collection of semen from roosters. The technique involves restraining the male and gently stroking the back of the bird from behind the wings towards the tail with firm rapid strokes. The male responds with tumescence erection of the phallus, at which time the handler gently squeezes the cloaca expressing semen through the external papillae of the *ducti deferentis* and collecting the semen into a vial’. Donoghue and Wishart (2000) reported that for turkeys, the technique is adapted by massaging the area around the cloaca before milking the semen. Various adaptations are also made for species such as waterfowl, which have penis-like copulatory appendages (Donoghue and Wishart 2000) and nondomestic species which require additional restraint (Gee, 1995). The proximity of the cloaca increases the likelihood of obtaining semen contaminated with faeces, urates, and bacteria that are detrimental to semen quality. Other authorities have adopted the procedure started by Burrows and Quinn (1937) with or without modification (Rekwot, 2002; Christensen 2005; Zaharaddeen *et al.*, 2005; Obidi *et al.*, 2008; Yahaya *et al.*, 2013).

2.9 Semen Evaluation

Semen evaluation is one of the important semen procedures and has been extensively discussed by several authorities. In the 60's and 70's inseminations were based on semen volume per dose, that is, certain amount of semen volume was inseminated without regard for sperm number. In the 70's and 80's inseminations were made based on number of spermatozoa per dose, and from the late 80's into the 90's inseminations were based on the number of viable spermatozoa per dose. These changes occurred obviously because 0.05 ml of diluted semen from several different semen samples

will not contain the same number of sperm. Tom-to-tom (pen-to-pen) differences and differences between semen collectors affect sperm concentration (Bakst, 1993).

Knowledge of sperm concentration helps to provide a basis for calculating the number of sperm per insemination and a measure of semen quality. Furthermore, by inseminating known numbers of sperm, AI protocols calling for an increase in the number of sperm to be inseminated at specified intervals during the egg production season can be used with some degree of reliability (Baskt and Long, 2010). With the advent of the ethidium bromide dye exclusion test, the determination of sperm viability became a relatively rapid and reproducible procedure. Once the sperm concentration and percentage of non-viable sperm are known, the volume of semen necessary to inseminate a predetermined number of viable sperm can be simply calculated. This appears to be beneficial with fresh semen and semen held less than 6 hours. However, adjusting sperm numbers to compensate for nonviable sperm following storage for 18 hour or longer is questionable (Bakst, 1993). Based on another sperm viability test (sperm stress test), about 50% of the sperm are nonviable (presumed dead or senescent) after storage for twelve hours (Bakst, 1993).

Commercial animal production relies on two basic processes to improve economic value: genetic selection/crossbreeding and production of progeny (multiplication).

Genetic selection is used to improve the nuclear or parental stocks, whereas effective reproduction in a multigenerational multiplication scheme (poultry, swine) or direct application (cattle) is essential to increase the number of progeny produced with desired genetic traits (Hartmann, 1988).

Reproductive effectiveness, that is, maximum number of offspring from each mating combination, not fertility, is the key element to the success of the overall production program. Unfortunately, animals that have been genetically selected for increased

growth or yield usually exhibit reduced reproductive performance (Reddy and Sadjadi, 1990; Reddy, 1995). The goal of identifying males with fertility above or below the means of their cohorts is not novel. A variety of assays have been used for more than 80 years to evaluate sperm (or semen) quality, classical papers before the era of computer include Williams (1920). Methods of semen evaluation rely on: evaluation of individual spermatozoa within a population, as reflected in percentage of motile or morphologically normal sperm cells, assessed by manual or computerized methods, or by detailed evaluation of large numbers of sperm via flow cytometry. The method also rely on average parameters for a population of cells by a host of biochemical approaches. Predictive use of most assays is limited, in part because for successful fertilisation, many attributes must operate correctly, and a defect in any one yields a sperm incapable of fertilizing an oocyte (Bakst and Cecil, 1996).

Indeed, fertilisation is the end-product of a "linear, obligatory, lock-step series of events" in which inappropriate expression of any single event causes lower-than-normal fertility (Hammerstedt, 1996). Any successful assay, then, must probe a combination of two or more important attributes or an individual attribute with global effects. General concepts of semen analysis for use to predict fertility within human and animal populations have been extensively reviewed (Amann *et al.*, 1993 ; Saacke *et al.*, 1994). Despite the availability of traditional measures of semen quality, the poultry industry has neither used reproductive technologies consistently nor improved fertility of pedigreed populations through careful analysis of males (Reddy and Sadjadi, 1990).

The test for live and dead sperm is achieved through a number of staining techniques; both live and dead stains are used for these techniques. The live stains include SYB 14 and Calcién AM and the dead stains include eosin and propidium (Sotirov *et al.*,

2002). The nigrosin/eosin (N/E) staining is much simpler and easier to use. The N/E staining procedure is based on the principle that live, viable sperm cells have intact cell membranes capable of excluding the eosin stain. Conversely, dead (non-viable) sperm cells possess permeable cell membranes that permit the eosin to stain the cells. The nigrosin component of this procedure provides a blue background in the smear to enhance the contrast of the ivory white, unstained "live" sperm cell, as well as the pinkish or magenta, stained "dead" sperm cell. Based on the N/E staining procedure, a good fresh semen sample from most breeder toms or broilers will have more than 90% live and morphologically normal sperm (Bakst and Long 2010).

The most widely used test of sperm quality from the initial stages of AI development until the present time has been the assessment of the proportion of normal, progressively moving sperm, thus a good microscope is the key (Foote, 2001). In addition to examining sperm with bright field microscopes, differential interference contrast microscopes, multiple stains, flow cytometry, and computer assisted sperm analysis (CASA) have contributed to improved quantification of sperm motion (Graham, 1978; Salisbury *et al.*, 1978; Garner *et al.*, 1997; Foote, 1998). Assessment of semen samples through parameters such as ejaculate volume, semen concentration, motility and morphology has been carried out in different species of animals like bulls (Rekwot *et al.*, 1988; Rekwot *et al.*, 1987), rams (Jibril *et al.*, 2011), in boars (Louis *et al.*, 1994), in rabbits (Louis *et al.*, 1994), in cockerels (Perry, 1960 and Etches, 1996), toms (Burke, 1984; Cecil and Bakst, 1988; Bakst, 1990; Chritensen, 2005; Zaraddeen *et al.*, 2005; Yahaya *et al.*, 2013), and drakes (Ghonim *et al.*, 2010).

2.10 Semen Diluents

Diluents are buffered salt solutions used to extend semen, maintain the viability of spermatozoa *in vitro*, and maximize the number of hens that can be inseminated.

Semen extension is important since poultry (especially the tom) semen is viscous and highly concentrated, containing 6-12 billion spermatozoa/mL. Semen diluents are based on the biochemical composition of chicken and turkey semen (Lake, 1995). Glutamic acid, the most prominent anionic constituent of avian seminal plasma, became a standard component of diluents (Donoghue and Wishart, 2000). There are many diluents available for poultry semen, both published recipes and commercially available products. Several investigators have compared the composition of various diluents and summarized fertility data across studies (Howarth, 1983; Bakst, 1990; Bootwalla and Miles, 1992). It is evident from the reviews that there is no standard diluent for poultry semen, and that studies are so variable in experimental design including time of insemination, vaginal depth and frequency of AI, number of spermatozoa inseminated and duration of fertility analyzed that discerning the benefits of different diluents is difficult. There are basic characteristics common to nearly all diluents; factors to maintain pH, osmolarity and provide energy source for spermatozoa (Christensen, 2005). The pH of a diluent can affect the metabolic rate and motility of spermatozoa. Buffering agents, consisting of a mixture of an acid and its conjugate base are formulated into a diluent to limit changes in pH. These usually include the mixture of phosphates, citrates and or organic Zwitter ionic molecules such as N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES), and N-tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES), in poultry diluents. Zwitter ionic molecules are important in diluents for avian semen because of lactic acid build-up, which can lower the diluent pH with increased storage time (Christensen, 2005).

Chicken and turkey spermatozoa can tolerate a pH range of 6.0–8.0 (Bogdonoff and Schaffner, 1954; Van Wambeke, 1977). The motility and metabolic rate of

spermatozoa can be altered by diluents' pH. For example, low pH may reduce motility, lactic acid production and oxygen uptake in avian spermatozoa, whereas high pH increases metabolic rates *in vitro* (Bogdonoff and Schaffner, 1954). Lake and Ravie (1979) demonstrated that pH of 6.8 and 7.1 improved survival over diluents with pH of 5.8 and 7.4 in an experiment, where chicken semen was stored for 24 hours. In another experiment with turkey semen stored for six (6) hours *in vitro*, higher fertility resulted when diluent pH was 6.5 compared to 7.5 (Donoghue and Wishart, 2000). Poultry spermatozoa can maintain fertilizing ability in diluents with osmolarities ranging from 250 to 460 mosM/kg H₂O. When spermatozoa are placed in a solution of low osmolarity, the net movement of water into the sperm cells causes them to swell. In a hyperosmotic solution, spermatozoa lose water and shrink (Bakst, 1980). This physical property is the basis of commonly used spermatozoa stress tests. In hypo osmotic conditions spermatozoa display increased incidence of bent necks (Donoghue and Wishart, 2000), a defect frequently found in diluted chicken and turkey semen, which is negatively correlated with fertility. Giesen and Sexton (1982) observed a disappearance of turkey spermatozoa over an 18-h storage period suggesting that spermatozoa are swelling and bursting *in vitro*. These investigators hypothesized that hypertonic diluents could reverse the swelling and improve survival *in vitro*.

The physiological differences and metabolic requirements of spermatozoa from different species are important to the development of diluents and storage systems for poultry semen. Chicken spermatozoa are metabolically competent in aerobic and anaerobic environments *in vitro*. In contrast, turkey spermatozoa require high levels of oxygen to survive (Sexton, 1974; Wishart, 1981). Aerobic metabolism necessitates the aeration of semen which is generally accomplished by placing diluted semen in a

flask to maximize surface to volume ratio and placing the flask on a rotary shaker. More recently, other methods to maximize and control oxygen supply to semen have been investigated. These include the addition of perfluorochemicals, compounds that chelate oxygen (Thurston *et al.*, 1994), and the development of environmentally-controlled capsules that regulate temperature and oxygen concentration (Donoghue and Wishart, 2000).

2.11 Semen Storage

Freshly collected chicken semen was among the first type of semen to be frozen (Polge *et al.*, 1949). However, cryopreserved poultry sperm are less fertile and freezing poultry sperm is still under development (Baskett and Long, 2010).

Chicken and turkey semen begin to lose fertilizing ability when stored more than 1 hour (Christensen, 2005). However, Christensen (2005) reported that liquid storage (4°C) of turkey and chicken semen can be used to transport semen and maintain spermatozoal viability for approximately 6-12 hours. Cryopreserved rooster sperm exhibited a high degree of motility, a greatly reduced fertility, and a significant decrease in the number of sperm trapped in the perivitelline membrane (Alexander *et al.*, 1993).

2.12 Frozen Semen

Freshly collected chicken semen was among the first type of semen to be frozen in England (Polge *et al.*, 1949), Glycerol soon was found to be useful for bull sperm. Many researchers had tried to freeze bull semen. The researches had focused on using sugars as cryoprotectants, but they did not lead to successful results (Foote, 2001). The basic medium used by Polge, *et al.*, (1949) was the original yolk-citrate extender (Foote, 2001) plus glycerol. Other workers developed whole milk-glycerol as a good medium to cryopreserve bull sperm (O' Dell and Almquist, 1957). Tris-buffered egg

yolk-glycerol also provided excellent protection for sperm either frozen or unfrozen (Davis *et al.*, 1963; Foote, 1998). This became the most commonly used medium worldwide for cryopreservation of bull sperm and sperm from several other species (Iritani, 1980). Fertility still is lower in many cases, but cows can be inseminated at a fixed time without detection of estrus (Nebel *et al.*, 2000). Rowson (1971) predicted that AI, combined with superovulation, synchronization of estrus, and manipulation of embryos would lead to major advances in animal production beyond the use of AI alone. Other authors have also made significant contributions in the areas of semen storage. Blesboi and Raviers (1992); Long and Kramer, (2003), and more recently Long *et al.*, (2003) reported that researchers are exploring potential ways of prolonging turkey semen by way of functional genomics through the use of Serial Analysis for Gene Expression (SAGE).

Evaluation of the post-thaw survival is important when considering frozen semen (Donoguhe and Wishart, 2000). Saacke and Marshall (1968), using differential interference contrast microscopy, pioneered the use of wet-smears of unfixed bull sperm to determine percentage intact acrosomes based on the presence or absence of the acrosomal (apical) ridge. Techniques used for evaluating sperm morphology have been reviewed (Barth and Oko, 1989; Foote, 2001). These include microscopy in wet smears, the use of the membrane impermeable dye eosin (eosin-nigrosin test), exposure to a hypo-osmotic saline solution (HOS-test) and the use of single or multiple fluorophores to study sperm morphology.

2.13 Reproduction in the Turkey Hen

The reproductive tract of the hen is suspended in the body cavity by a ligament that attaches the tract throughout its entire length to the dorsolateral part of the body cavity. In most birds only the left oviduct is present and functional. The oviduct

comprises the infundibulum, which is the site of fertilisation that engulfs the ovulated ovum. It contributes in the formation of the chalazae and sperm storage (Hafez and Hafez, 2000). The magnum is the longest part of the oviduct and functions majorly in albumen production (Taylor, 2003). The isthmus region of the oviduct is separated from the magnum by a narrow translucent area which does not possess any tubular glands and contributes to the formation of the egg membranes and about 80% of the time required for egg formation in the oviduct is spent in the uterus, or shell gland region (Taylor, 2003). The mucosa of the isthmus is folded into primary and secondary ridges which are aligned longitudinally and the tubular glands in this region have secretory cells which are believed to secrete the cores of the fibres that make up the shell membrane, while the secretory cells of the epithelium secrete the mantle that surrounds them (Etches, 1996). The egg spends 18 to 22 hours in the shell gland of the oviduct, absorbs approximately 15 g water in the chicken, and exchanges several electrolytes, including sodium, potassium and chlorine. This gland contains several types of secretory cells in both the epithelium and the tubular gland. Water-containing electrolytes is absorbed by the egg in this region and, it decreases with an increase in the rate of shell calcification (Taylor, 2003).

2.14 Artificial Insemination

Artificial insemination (AI) in poultry is the process of collecting semen and manually placing the semen into the female tract. This technique is performed to avoid the spread of venereal diseases by natural mating and to increase the dissemination of genetic material to a large number of birds. Long-term storage of cockerel semen has been reported to be achieved by using a suitable cryoprotectant and storage at -196°C (liquid nitrogen). The discovery of glycerol's cryoprotective properties pioneered the success of modern cryobiology and led to the development of semen cryopreservation

for a wide range of species. Despite the fact that this scientific break-through was accomplished with rooster semen (Polge, 1951), the overall fertility rates with frozen/thawed poultry semen are highly variable and not reliable enough for use in commercial production or preservation of genetic stocks. Moreover, significant differences exist among the commercial poultry species (turkey, broiler-type chicken, and layer-type chicken) in terms of the viability and functionality of sperm after cryopreservation. In particular, the fertility rates from frozen/thawed turkey semen consistently have been lower than cryopreserved chicken semen (Nelson *et al.*, 1980; Sexton, 1981; Kurbatov *et al.*, 1986; Schramm and Hubner, 1988; Wishart, 1989). Cryoprotectant types and packaging methods, as well as freezing and thawing rates, for avian sperm cryopreservation have been reviewed, (Lake, 1986; Hammerstedt and Graham, 1992; Donoghue and Wishart, 2000).

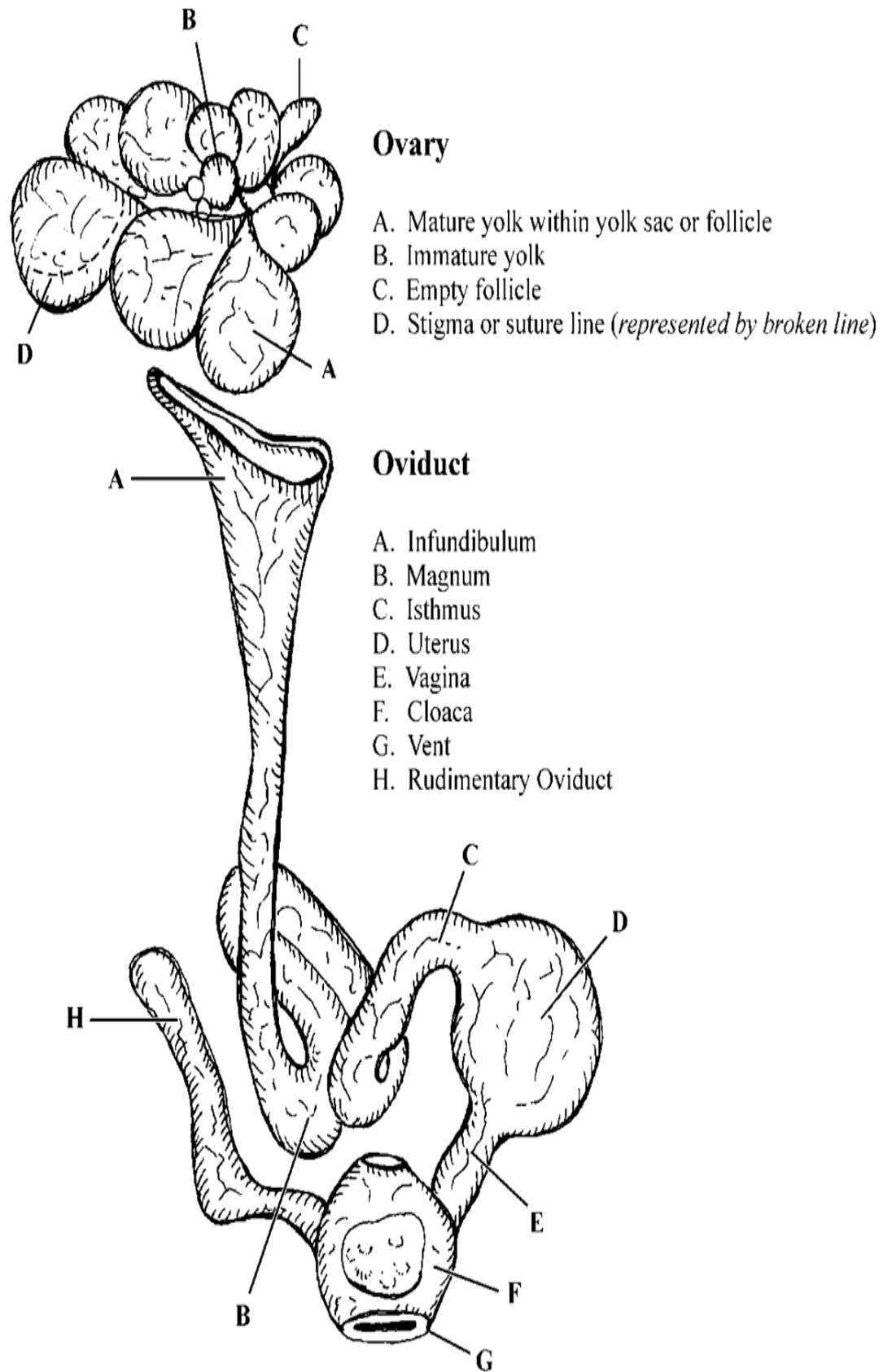


Figure 2.2: Reproductive system of the turkey hen
Source: Banerjee (2011)

2.14.1 Insemination techniques

The most reliable and successful routine for insemination of poultry, is by depositing semen directly in the mid-vaginal area. To achieve this, the vaginal orifice must be everted, by using gentle abdominal pressure (Cole and Cupps, 1977).

2.14.1.1 Intra-Peritoneal Insemination

This technique of AI is not reliable and has been used periodically for many years. In this technique a sharp needle is punched through the abdominal wall and the cannula inserted to deposit semen in the region of the ovary (Cole and Cupps, 1977).

2.14.1.2 Vaginal Insemination

This is the most commonly used AI procedure and two people are required for this operation. The placement of semen into the hen's reproductive tract is accomplished by inverting the cloaca of the hen to expose the opening of the vagina. The insemination pipette is inserted and sperm cells delivered to a location near the SST. The cloaca is everted by holding the thighs of the hen between the thumb and forefinger, while the body rests in the palm of the left hand (Etches, 1996). Hens have to be inseminated for two consecutive days for the first time, and thereafter once a week, if fertile eggs are required. Cockerel semen has a limited life outside the body, and must be deposited in the hen within 1 hour of semen collection from the cockerel, in the case of fresh semen (Etches, 1996).

It is best to inseminate hens in the afternoon (14:00 and 16:00) as in the morning hens may have an egg in the oviduct, making difficult it for the sperm to swim up to the ovary. Eggs are normally fertile after the second day of insemination, and the fertility period can remain for two or more weeks (Martin, 2004). It has been shown that hens inseminated with fresh semen produce more fertile eggs than hens inseminated with frozen/thawed sperm (Blanco *et al.*, 2000). The fertility of the eggs can also be

influenced by the transport of sperm. Hens that lay eggs intensively lay fewer infertile eggs and have greater duration of fertility, compared to poor layers (Lake, 1983).

The technique currently used for AI in poultry was developed in the 1930s and involves applying pressure to the hen's abdomen and everting the vaginal orifice through the cloaca (Burrows and Quinn, 1937). This procedure is also referred to as cracking, venting or everting the hen. Semen is deposited 2–4 cm into the vaginal orifice concurrently with the release of pressure on the hen's abdomen. Insemination is accomplished with straws, syringes or plastic tubes. In large scale, commercial operations, automated semen dispensers and individual straws loaded with a set AI dose are commonly used.

2.14.2 Behaviour of sperm in the oviduct of the hen

Avian spermatozoa can survive in the female reproductive tract and are capable of fertilizing eggs for a long period of time in many species. Van Dremmelen discovered anatomical structures associated with sperm storage (Hatch, 1983). These structures are called sperm nests, sperm glands, sperm storage tubules or sperm-host glands, and they occur in the infundibulum and the uterovaginal junction (King *et al.*, 1999). Spermatozoa introduced by copulation or AI are stored in these organs and retain their fertilizing ability. A model accounting for the mechanism of sperm storage was deduced from the behavior of motile sperm *in vitro*, sperm storage tubule (SST) histology, and the SST epithelial cell ultra structure. Sperm residing in the SST were considered to be immotile. It is, however, likely that residence depends upon the sperm moving against the current generated by the SST epithelial cells (Froman and Feltmann, 2005).

Avian spermatozoa are motile at a body temperature of 41°C for an interval of days to weeks following ejaculation. How the sperm enter, survive, and exit these sperm

storage tubules however is still experimental. Movement of sperm to the uterovaginal region is fast, and only viable sperm enter the sperm storage tubules. Current evidence suggests that the release of stored sperm is episodic, although it was first thought to be associated with oviposition. Movement of sperm through the oviduct is achieved by smooth muscle contractions and/or ciliary activity (Hafez and Hafez, 2000).

The sperm in mammals spend a relatively short time in the female tract, while in chickens and the turkey sperm can spend a much longer period of time in the oviduct before fertilizing the egg yolk cell - up to 32 days in the chicken and 70 days in the turkey (Hafez and Hafez, 2000). The process of prolonged sperm storage is not known; it is thought to include a reversible suppression of respiration and motility of the sperm, as well as stabilization of the plasma membrane and maintenance of the acrosome (Tabatabaei *et al.*, 2009). According to Mauldin (2000), spermatozoa are released from the sperm storage tubules to fertilize the sequentially ovulated ova at regular intervals. After release the spermatozoa are taken to the ovum by contraction of the hen's oviduct, and sperm motility may no longer be critical. Within 5 to 10 minutes after ovulation, spermatozoa reach the germinal disc on the surface of the ovum.

The spermatozoa that make contact with the perivitelline layer of the ovum undergo an acrosome reaction and, presumably, by the action of the trypsin-like enzyme acrosin, hydrolyze the perivitelline layer. Theoretically, only one sperm fertilizes the ovum, but polyspermy has been observed in the hen ovum with many holes hydrolyzed in the perivitelline (Hafez and Hafez, 2000).

Several factors influence fertility. After AI, semen quality can be affected by age of the tom or rooster, lighting schedule, season, body weight, and diet, as well as the semen collector (Sexton, 1986). Timing of AI is important, and is usually performed

in the late afternoon to minimize the number of hens with hard-shelled eggs in the shell gland. In turkeys, Brillard and Bakst (1990) demonstrated that sperm numbers in the SST of hens inseminated before the onset of lay was twice that of hens inseminated at the beginning of egg production. Therefore, turkey hens are generally inseminated before they begin to lay, usually 14 to 17 days after increased lighting for stimulation of egg production. In chickens, AI is usually initiated when 15% to 20% of the hens are in egg production (Donoghue and Wishart, 2000).

2.15 Gonadal and Extragenadal Sperm Reserve

Morphometric analysis on the testis of any species or breed is necessary in assessing and estimating quantitative changes in testicular components and spermatogenic functions, arising from factors such as age, season, temperature, disease (Egbunike *et al.*, 1976) and nutrition (Bielli *et al.*, 1997). Knowledge of the basic morphometric characteristics of the reproductive organs is mandatory for assessment and prediction not only of sperm production, but also of the storage potential and fertilizing ability of the breeder male. A method was developed for the estimation of the gonadal and extragenadal sperm reserves in bulls (Almquist and Amann, 1961). This method was modified and adapted to determine gonadal and extragenadal sperm reserves in various species: rats (Adedapo *et al.*, 2003; Olukole *et al.*, 2010), goats (Bitto and Egbunike, 2006; Ugwu, 2009), rabbits (Ogbewu *et al.*, 2009; Amao *et al.*, 2012), camels (Ibrahim *et al.*, 2012), stallions (Gebauer *et al.*, 1974), rams (Bielli *et al.*, 2000; Gundogan, 2006), pigeons (Orsi *et al.*, 2007), domestic fowls (Obidi *et al.*, 2008) and turkeys (Cecil *et al.*, 1988). Cecil *et al.* (1988) reported the following values for exotic breed of turkey;

Gonadal sperm reserve, $122-127 \times 10^6/g$, extragenadal reserve for; epididymis = 58×10^6 cells (ejaculated) and 204×10^6 cells (rested), *vas deferens* = 3160×10^6 cells

(ejaculated) and 10320×10^6 cells (rested), Total extragonadal reserve = 3248×10^6 cells (ejaculated) and 10524×10^6 (rested).

2.16 Sperm Penetration Assay

Avian fertilisation has a distinctive temporal relation to oviduct transit (Bakst *et al.*, 1994; Wishart, 1995), wherein fertilisation occurs only in the infundibulum and before subsequent processing of the egg during oviduct passage. A further distinctive feature is that the target for sperm, the germinal disc, constitutes only about 2% of the total surface of the freshly ovulated ovum, entering the infundibulum. A critical observation first made was that some sperm cells do not penetrate the ovum at fertilisation, but remain trapped in the outer perivitelline membrane (PVM) (Bobr, 1964). Then, studies, (Wishart, 1995; Wishart, 1987; Staines *et al.*, 1997), clearly demonstrated a critical relationship. There was a high correlation between the number of sperm trapped in the outer PVM of "the other 98% of the oocyte surface" of the first egg after mating or AI, and probability of subsequent eggs being fertile, and days over which fertilized eggs would be laid (the duration of fertility).

Similar observations were made for sperm-egg interactions in other animals; the trapped sperm is often referred to as "accessory sperm" within the *zona pellucida*, in cattle and humans (Davis *et al.*, 1963; Alexander *et al.*, 1993).

2.16.1 *In vivo* and *in vitro* sperm penetration assay

At the time of fertilisation, the ovum is enveloped by the inner-perivitelline layer (PL) which is a thin, fibrous investment (Bakst and Dymonds, 2013). The spermatozoa digest their path through the PL in order to reach the germinal disc for syngamy and fertilisation of the ovum. The sperm penetration assay provides a means to quantitatively determine the number of sperm penetration sites (sperm-holes) in the PL overlying the germinal disc. When performed with freshly laid eggs, it can be used

as a predictive measure of fertility (Bakst and Long, 2010). The original procedure was developed for chicken eggs (Bramwell *et al.*, 1995), but later adapted for turkey eggs (Donoghue, 1996). Several works have been carried out to demonstrate the applicability and advantage of the assay in turkeys (Barbato *et al.*, 1998; Wishart and Staines, 1998) and in drakes (Al-Daraji, 2001).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Study area

The study was conducted in the Department of Theriogenology and Production, Ahmadu Bello University Zaria, situated in the Northern Guinea Savannah zone of Nigeria, between latitudes 11° 15'33" N and between longitude 7° 6'49.89" E at an elevation of 646 m above sea level. The mean annual rainfall in the area is 1100 mm, lasting from May to October (816 mm/month). Mean daily temperature during the wet season is 25°C, and mean relative humidity is 72%. The dry season lasts from November to April, the mean daily temperature ranges from 14 to 36°C and the relative humidity is 20-30% (Anonymous 2014).

3.1.2 Study animals

Fifteen turkey toms 30-34 weeks and weighing 3.5 – 4.5kg and 9 turkey hens aged 20-24 weeks and weighing 2-2.5 kg were bought from Wurno town, Wurno Local Government Area of Sokoto State for the study. The turkeys were caged individually in improvised cubicles with the dimension of 1.5 metres x 1.5 metres. The feed used for this study was formulated in collaboration with the Department of Animal Science, Faculty of Agriculture, Usmanu Danfodiyo University, Sokoto (Table 3.1). It was compounded at AMBUVET CONSULT, Kaduna, while the proximate analysis was done at the Department of Chemistry, Faculty of Science, Usmanu Danfodiyo University, Sokoto (Table 4.1). Nutrient requirements were based on the NRC (National Research Council) 1984 (Cited by Firman, 1993).



Plate I: A tagged tom used in the experiment.

Table 3.1: The formulated feeds

S/No	Ingredients (kg)	Low 12% CP	Moderate 16% CP	High 20% CP
1	Maize	66.0	56.50	43.80
2	Groundnut Cake	5.50	17.40	29.30
3	Wheat Offal	23.70	21.30	22.10
4	Limestone	2.40	2.40	2.40
5	Bone meal	1.80	1.80	1.80
6	Premix	0.25	0.25	0.25
7	Salt	0.30	0.30	0.30
8	Methionine	0.01	0.01	0.01
9	Lysine	0.01	0.01	0.01

The feeds were isocaloric

3.2 Methodology

3.2.1 Experimental design

Twenty four (n = 24), apparently, healthy turkeys, comprising 15 toms and 9 hens were used in this study.

The turkey toms were randomly placed in three groups (Group 1, Group 2 and Group 3) of five, and were tagged appropriately, while the hens were placed in three groups (Group F1, Group F2 and Group F3) of three.

3.2.2 Feeding and water supply

The tom groups received 400 g/bird /day of feed containing different levels of crude protein, viz: 12% CP, 16% CP and 20% CP for Groups 1, 2 and 3 respectively. The females were fed commercial layer mash (16% CP). All the birds were given access to water *ad libitum*. The live weights of all the turkeys were determined weekly throughout the period of the study using a hanging scale (Metler Inc. USA) (Plate II). They were acclimatized for a period of two weeks during which the males were trained for semen collection.



Plate II: Weighing of a turkey tom using a weighing scale.

3.2.3 Semen collection and evaluation

Semen was collected twice weekly (two ejaculates per collection) using a modified abdominal massage technique (Bakst and Long, 2010) and evaluated for; volume, colour, motility (gross and individual), concentration, percentage live and dead cells and morphology.

Three people were involved in this procedure; the restrainer, the massager, the collector. Each tom was placed in the armpit of the restrainer. The semen collector firmly "massaged" the tom's abdomen with one hand while concurrently stroking firmly the back and tail feathers with the other hand. Within few seconds, the phallus enlarged slightly (phallic tumescence) and everted, the massager then shifted his hands to straddle the cloaca. The thumb and forefinger of the hand situated just above the cloaca squeezed it gently downward and inward while simultaneously the thumb and forefinger of the hand situated just below the cloaca squeezed upward and inward (Plate III). This action, referred to as a "cloacal stroke", resulted in a partial ejaculation and this was repeated a second time to complete the ejaculation process. The semen was then collected in a graduated collection tube and the volume recorded. The same set of procedures was used for semen collection before and during treatment.

All the hens were placed on commercial layer mash (16% CP) and were inseminated weekly with a dose of 100×10^6 sperm cells (Christensen, 2005). The eggs laid by the hens were used for the *in vivo* sperm penetration assay. An interval of two weeks was given between the first insemination and egg collection.



Plate III: Turkey tom restraint and semen collection.

3.2.3.2 Semen Evaluation

Semen samples collected were evaluated as described by Baskt and Long (2010) with modifications.

Volume: The semen was collected into a 1-ml graduated tube and the volume recorded immediately (Plate IV).

Mass motility: Microscopic examination for wave pattern (gross sperm motility) was determined by placing a drop of raw undiluted semen on a pre-warmed slide and covered with cover-slip; it was viewed under a microscope at x4 and x10 objectives and motility was estimated by subjectively assessing the wave pattern.

Individual motility: This was determined using diluted semen samples. Semen samples were diluted to ensure single layer of sperm cells for ease of counting. This was achieved by adding three drops of Dulbecco's Modified Eagle's Media to one drop of raw semen. A drop of diluted semen was then placed on a grease free microscope slide and viewed at x 40 objective. Sperm cells progressively moving forward were counted as percent motile cells.

Concentration: Neat semen was pre-diluted 1:2 with Egg yolk-Citrate extender. Then 10 µl of the extended semen was diluted in 10 ml of 3% NaCl, dilution factor of 1000. Next 10 µl of diluted semen was used to fill one chamber of haemocytometer and the spermatozoa were counted in five Thoma squares of the chamber (that is, four corner and the center squares). The concentration was then determined by the following equation;

Concentration (sperm cells/ml) = Number of sperm cells counted in the twenty five small squares x dilution factor x 10^4 .



Plate IV: Semen sample in a graduated collection tube immediately after collection.

Live sperm count: This was determined as described by Estes *et al.* (2006) with modification. A drop of semen sample was placed at the edge of a clean grease free glass slide and three drops of eosin-nigrosin stain were mixed with the semen. A smear was made from the mixture. Four hundred (400) sperm cells were counted using a hand counter under a light microscope at X100 magnification (oil immersion) when the slides were dried (Plates V and VI). The numbers of both live and dead cells were recorded and the percentage of each was calculated. The nigrosin-eosin solution used in this work contained potassium citrate 1.280 g, sodium glutamate 1.7351 g, sodium acetate 0.851 g, magnesium chloride 0.686 g, eosin 1.0008 g and nigrosin 5.0002 g (Lukaszewicz *et al.*, 2008).

Spermatozoa morphology: Morphology was determined by making a thin smear from a mixture of the semen sample and eosin-nigrosin on clean grease free glass slide (Plate V and Plate VI). Four hundred sperm cells were counted per slide using a hand counter under light microscope at X 100 magnification (oil immersion) (Estes *et al.* 2006). Five types of cells were recorded; normal cell, bent head, coiled tail, free head and macrocephalic head. The number of each was divided by four to get its percentage.

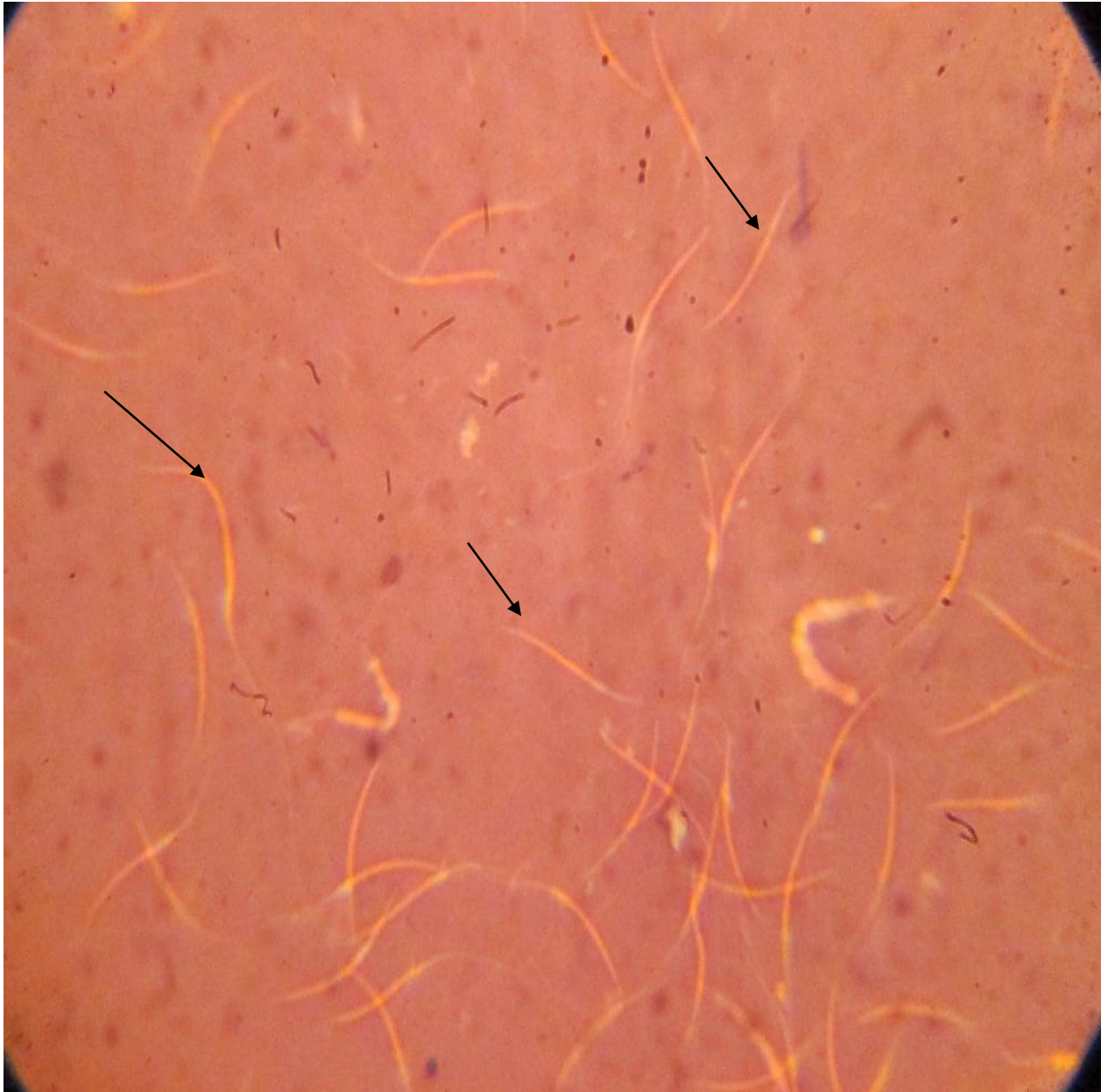


Plate V: A photomicrograph of eosin-nigrosin stained slide showing live spermatozoa. (Black arrows).

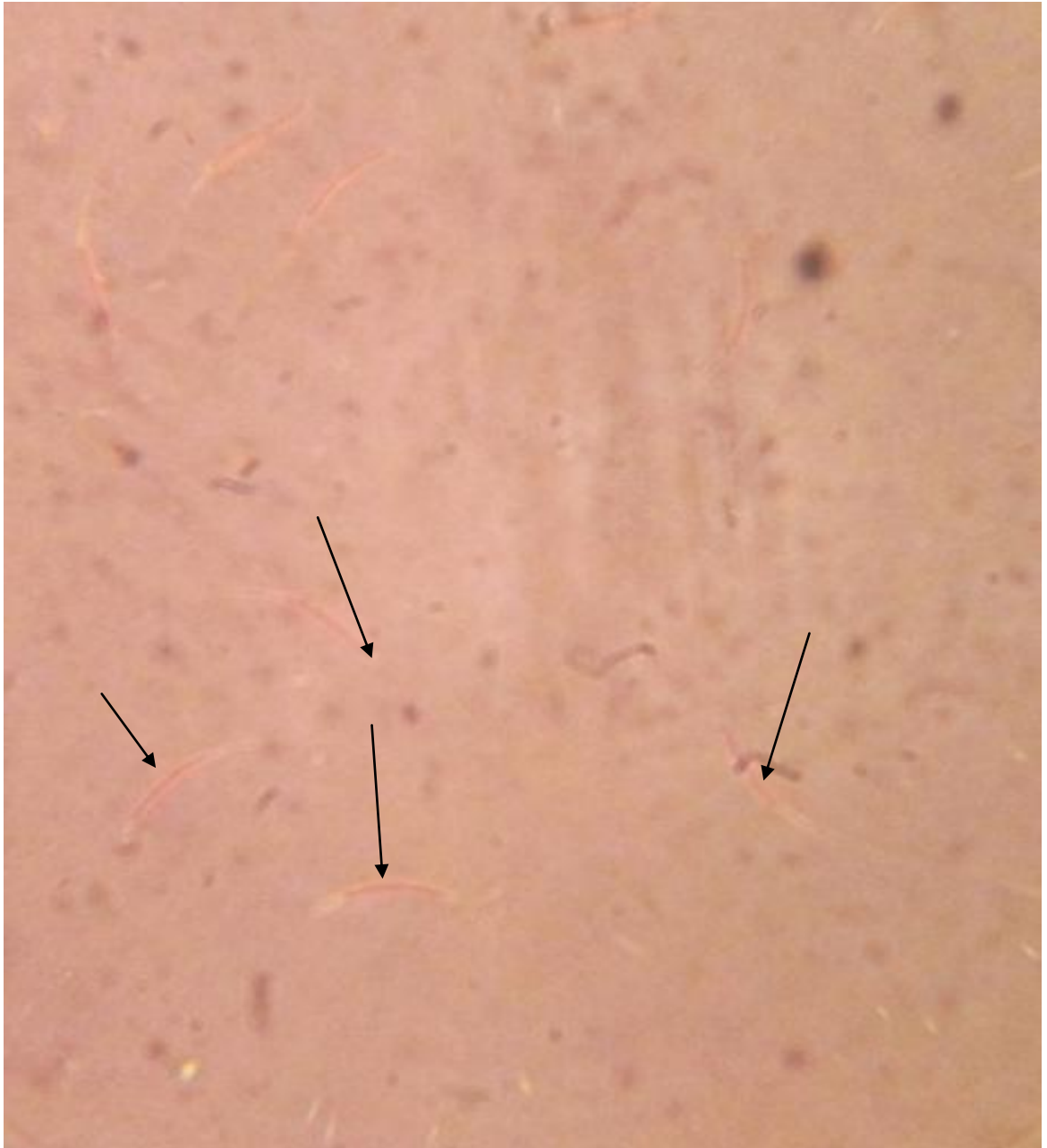


Plate VI: A photomicrograph of eosin-nigrosin stained slide with dead cell deeply stained (Black arrows).

3.2.4 Sperm penetration (SP) assay

3.2.4.1 Insemination of the Hens

All hens were inseminated weekly according to the method described by Sotirov, (2002). The hens were turned upside down, pressure applied to the right side of the abdomen until the vent everted (venting) and an insulin syringe containing the fresh diluted semen which was obtained from the toms was inserted to a depth of about 1.5-2 cm into the cloaca, and semen containing 100×10^6 spermatozoa was deposited in the vagina (Plate VII).

Number of sperm cells inseminated: At the start of the insemination the average semen concentrations were 5×10^9 for Group 1, and 6×10^9 for Groups 2 and 3.

In Group 1 100 μ l was diluted in 2.5ml of Dulbecco's Modified Eagles Medium (DMEM), each hen was thereafter inseminated with 0.5ml of the diluted semen. In Group 2 and 3, 83 μ ls were used instead.



Plate VII: A turkey hen being inseminated using a hypodermic syringe.

3.2.4.2 *In vivo* assay

Sperm penetration assay was carried out according to the method described by Baskt and Long (2010). The oviposited eggs (from inseminated hens) were broken and the albumen separated from the yolk. The yolk was then placed in a pan with the blastoderm positioned upwards. Excess albumen was removed by blotting with kimwipe and 2% NaCl solution was added; an area of 3.0 mm² of the perivitelline layer over the blastoderm was carefully cut and immediately rinsed in phosphate buffered saline to remove excess yolk material. The perivitelline layer was then placed on a glass slide and 4 drops of 3% formalin were added to fix the membrane and immediately decanted. The perivitelline layer was finally stained with Schiff's reagent. The holes were then counted under Datyson Biological microscope at a magnification of X 40.

3.2.4.3 *In vitro* assay

This is an *in vitro* sperm quality assay in which spermatozoa were co-incubated in a test tube with inner perivitelline layer (IPL) from fresh unfertilised eggs. The number of sperm holes per unit area of the IPL were then viewed under dark-field optics and quantified as a measure of sperm activity. The IPL samples for the assay were obtained from fresh eggs sourced from poultry layer unit of the National Animal Production Research Institute (NAPRI). They were separated from the outer perivitelline layer (OPL) by acid hydrolysis as described by Baskt and Long (2010). Briefly the procedure is described below.

Separation of IPL from OPL: This was done as follows:

- 1) The eggs were cracked open over a waste beaker retaining the yolk in one half of the shell.
- 2) The albumen was thoroughly decanted and the yolk isolated.

- 3) The isolated yolk was placed in a small dish and washed several times with 1% NaCl to remove any excess albumen. The adherent pieces of albumen were removed using blunt-ended forceps.
- 4) The yolk was placed the yolk in a 100-mL beaker containing 75 mL of 0.01 M HCl and the beaker placed in a 37 °C incubator for 1 hour.
- 5) After 1 hour, the beaker was removed from the incubator and the HCl was decanted taking care not to burst the yolk. The yolk was then transferred to a small bowl and the perivitelline layer (PL) bursted from the side with the aid of sharp forceps and the PL isolated.
- 6) The isolated PL was then placed in a petri dish containing 1 % NaCl and washed in several changes, until all yolk particles were removed; and the PL was then spread out in a petri dish, containing fresh 1% NaCl. The IPL was distinguished from the OPL by its thinner, more transparent, appearance. The two layers were then separated using sharp forceps and the isolated IPL was stored in a petri dish containing NaCl-TES at 5°C for the sperm egg assay (Bakst and Long, 2010).

3.2.4.4 Sperm-egg assay

- 1) Ten (10) µl of fresh semen were diluted in 1000 µl of NaCl-TES for Group 1 and 8µl parts of fresh semen was diluted in 1000 µl parts of NaCl-TES for Groups 2 and 3; the aliquots were stored at 40 °C in a shaking water bath for 45 min before assay.
- 2) A fragment of the isolated IPL approximately 0.5 cm x 0.5 cm square was cut and added to a vial containing 100 µl of NaCl-TES diluted semen in 1 ml Dulbecco's Modified Eagle Medium (DMEM). The mixture was incubated at 40°C for 5 min in a shaking water bath.
- 3) The incubated IPL was removed from the vial and washed in 1% NaCl.

- 4) The IPL was carefully spread onto a microscope slide using forceps avoiding wrinkles and then cover-slipped (Plate VIII).
- 5) The slide was then viewed immediately using dark phase microscopy at X 10 and X 40 objectives.
- 6) The number of holes per 3 mm^2 were counted according to the method described by Baskt and Long (2010) with modifications (Plate IX).

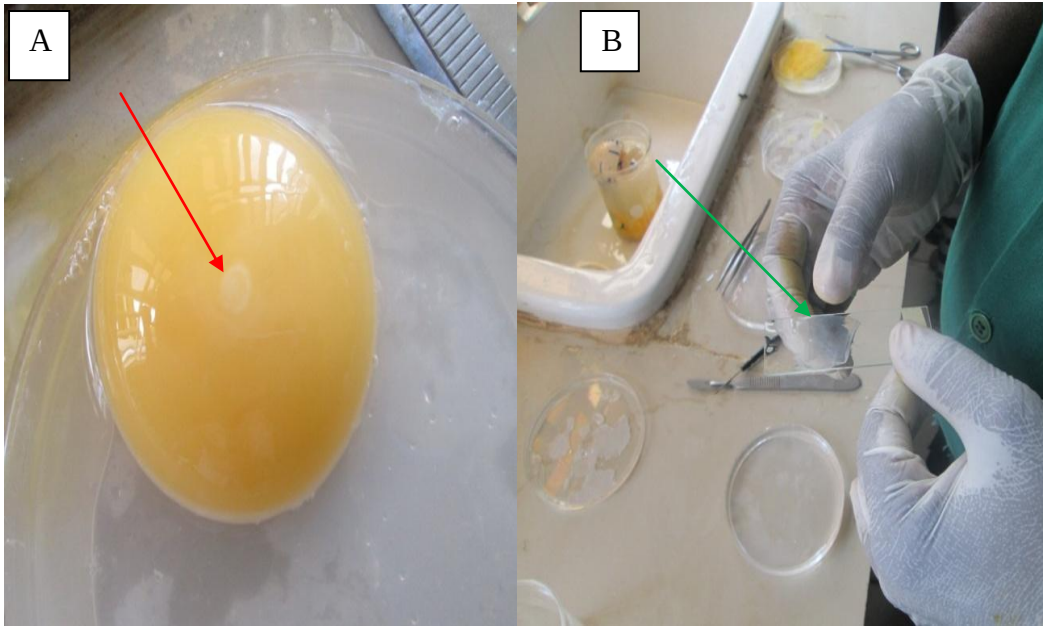


Plate VIII: Germinal disc on an egg yolk (A, red arrow), a section of PVM being used to prepare a slide (B, green arrow)

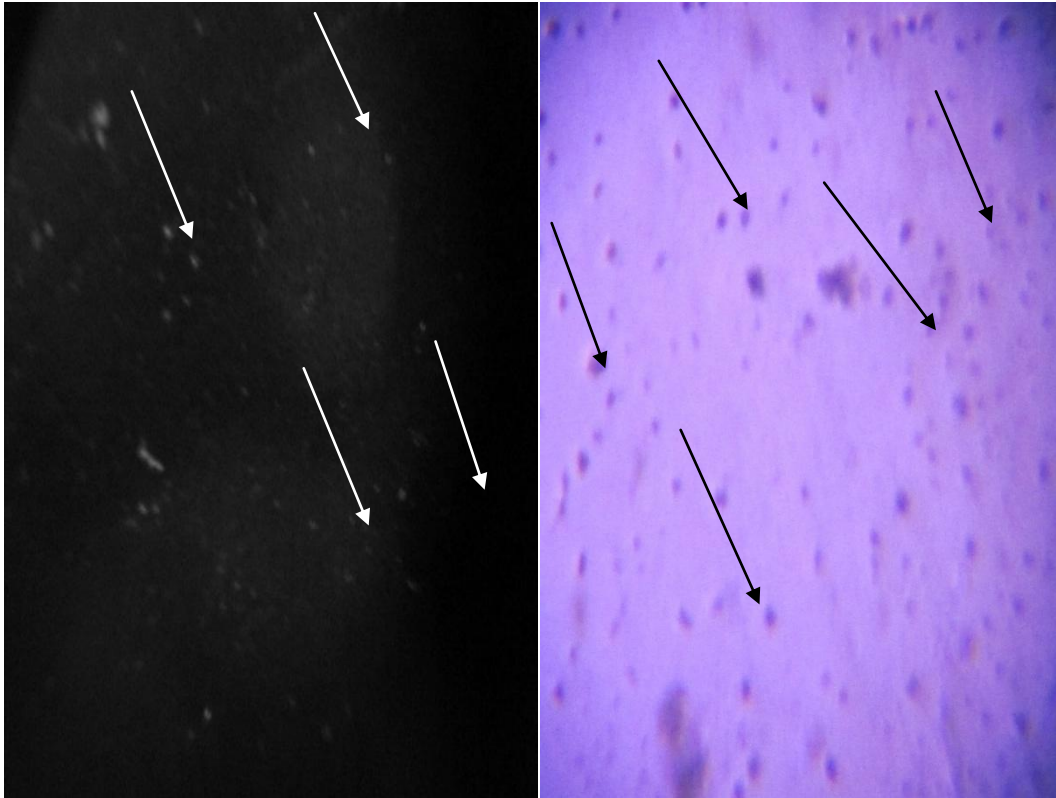


Plate IX: Sperm Penetration holes on a PVM layer section viewed at X40

3.2.5 Determination of gonadal and extra gonadal sperm reserves

Testicular, epididymal and *vas deferens* sperm reserves were determined using the homogenization haemocytometric technique (Obidi *et al.*, 2008) with modifications. Three toms from each group were sacrificed for this procedure. The left and right testicles were carefully removed, trimmed of extra tissues and measured; the weight, length and circumference were recorded (Plate X, Plate XI and Plate XII). After careful removal of the *tunica albuginea* with a scalpel blade, each testicle was weighed again and homogenized in 20 mls of physiological saline with an antibiotic (Streptomycin 1:20 v/v), using a mortar and pestle. The epididymis and *vasa deferentes*, were carefully removed, trimmed of extra tissues and measured; the weights, lengths and diameters were recorded. They were first minced with pair of scissors and each was homogenized in 10 mls of physiological saline containing the antibiotic. The homogenate volume was recorded and stored at 5 °C for 24 hours. They were mixed through shaking at intervals during the period of storage.

The homogenates were diluted 1:50 v/v using physiological saline. They were shaken for about a minute and filtered. Volumes of the filtrates were recorded and the sperm/spermatids reserves in the suspension were determined using Neubauer haemocytometer. Two counts were made for each suspension at a magnification of X 40. The number of spermatozoa counted was then multiplied by the dilution factor to obtain the number of sperm cells/ml of the homogenate; this was multiplied by the volume of the homogenate to obtain the total number of sperm cells in the homogenate.

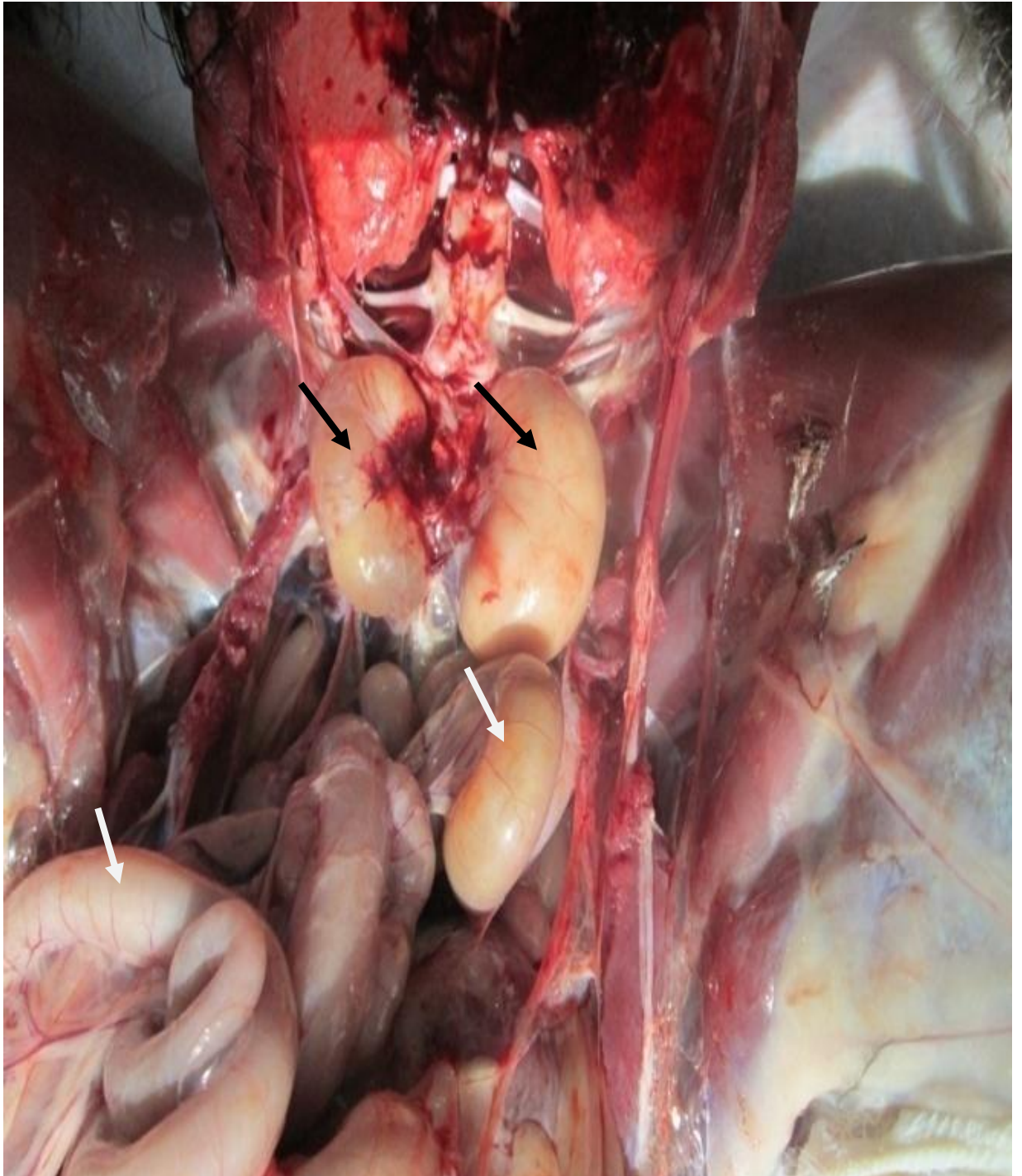


Plate X: A pair of testicles *in situ* (thick black arrows) and intestinal segments (thick white arrows).

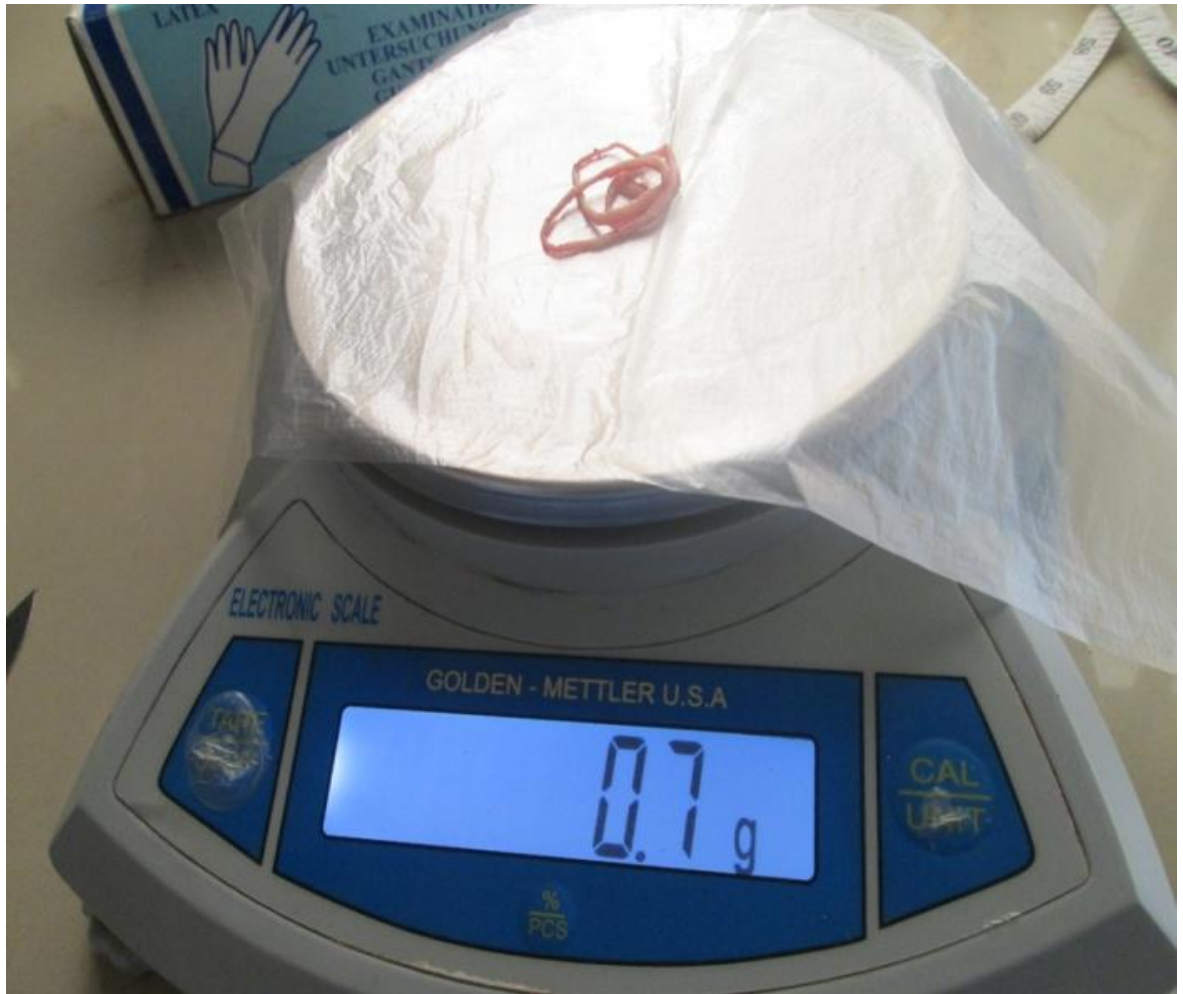


Plate XI: A *vas deferens* on an electronic weighing balance.



Plate XII: A *vas deferens* being measured using a measuring tape

3.2.6 Blood collection

Two (2) mls of blood was aseptically collected every week from the wing vein of each tom after the first semen collection of every week. The haemogram (complete blood count and packed cell volume) was determined in the haematology laboratory.

3.2.7 Statistical analysis

Statistical package “Graph pad prism version 6.0” was used for the analysis. Data are expressed as mean $\pm$ SEM (standard error of mean). The differences in the mean values of the semen parameters, sperm penetration holes and gonadal and extragonadal sperm reserves between the three groups were compared using Repeated Measure Analysis of Variance (ANOVA). Correlation matrix analysis was used to determine the relationship between live weights of the toms and their semen parameters.

CHAPTER FOUR

RESULTS

4.1 Proximate Analysis of Feeds

The result of proximate analysis of the feeds used are presented in table 4.1, the nutrients are within the acceptable ranges of NRC (Firman, 1993).

4.2 Live Weights and Semen Parameters

Live weights and semen parameters before and during treatments are shown in table 4.2 and 4.3 While the weekly trend for the parameters are presented in figure 4.1, 4.2 and 4.3 for live weights, semen volume and semen concentration respectively.

4.2.1 Live weights

Mean ($\pm$ SEM) body weight changes of toms fed varying levels of crude protein diets were 5.29 ± 0.25 kg, 5.39 ± 0.20 kg and 5.63 ± 0.22 kg for Groups 1, 2 and 3 respectively. The differences recorded between the groups did not differ significantly ($P > 0.05$).

The weekly trend observed in the live weights of the three groups did not reveal significant ($P > 0.05$) difference between all the groups.

4.2.2 Semen volume

Mean ($\pm$ SEM) semen volumes of the three groups of toms fed varying protein diets were $0.17^a \pm 0.01$ ml, $0.24^b \pm 0.02$ ml and $0.27^c \pm 0.02$ ml significant ($P < 0.05$) difference was observed between Groups 1 and 2, there was also a significant difference between Groups 1 and 3 but no significant ($P > 0.05$) difference was observed between Groups 2 and 3.

4.2.3 Weekly trend in the live weights

The weekly trend observed for the live weights of the three groups revealed a none significant differences between all the groups

4.2.4 Weekly trend in semen volumes

The weekly trend observed for the semen volumes of the three groups revealed significant ($P < 0.05$) difference between groups 1 and 2, and between groups 1 and 3 but there was no significant ($P > 0.05$) difference between groups 2 and 3.

4.2.5 Semen pH

The results (mean $\pm$ SEM) of the PH shown in Table 4.1 are 6.86 ± 0.06 , 7.03 ± 0.060 and 6.85 ± 0.07 . The differences observed between the three groups were not significant ($P > 0.05$).

4.2.6 Sperm motility

The results (mean $\pm$ SEM) of the mass motilities are $73.01^a \pm 1.34$ %, $79.09^b \pm 1.36$ % and $80.00^b \pm 1.19$ %. Significant ($P < 0.05$) difference was observed between groups 1 and 2 and also between groups 1 and 3. There was no significant ($P > 0.05$) difference between groups 2 and 3.

The results (mean $\pm$ SEM) of the individual motilities are $80.18^a \pm 1.29$ %, $85.58^b \pm 1.26$ % and $85.39^b \pm 1.12$ %. Significant ($P < 0.05$) difference was observed between groups 1 and 2 and also between groups 1 and 3. There was no significant ($P > 0.05$) difference between groups 2 and 3.

4.2.7 Semen concentration

The results (mean $\pm$ SEM) of semen concentrations of the three groups are $5.33^a \pm 0.428 \times 10^9$, $6.99^b \pm 0.56 \times 10^9$ and $7.77^b \pm 0.612 \times 10^9$. There was significant ($P < 0.05$) difference between groups 1 and 2. There was also a significant ($P < 0.05$)

difference between groups 1 and 3 but the difference between group 2 and group 3 was not significant ($P > 0.05$).

4.2.8 Weekly Trend in Semen concentration

The weekly trend observed for the semen volumes of the three groups revealed significant ($P < 0.05$) difference between groups 1 and 2, and between groups 1 and 3 but there was no significant ($P > 0.05$) difference between groups 2 and 3.

4.2.9 Live sperm count

Mean ($\pm$ SEM) counts of live spermatozoa are $76.81^a \pm 1.36$ %, $81.14^b \pm 1.32$ % and $82.80^b \pm 1.12$ % for groups 1, 2 and 3 respectively. Significant ($P < 0.05$) differences were observed between groups 1 and 2 and between groups 1 and 3, but there was no significant ($P > 0.05$) difference between groups 2 and 3.

4.2.10 Total sperm defect

The results (mean $\pm$ SEM) of the total sperm defects, are 18.43 ± 1.07 %, 17.31 ± 1.00 % and 17.00 ± 0.80 %. The difference observed between the three groups was not significant.

Table 4.1 Chemical Composition of the feeds (Result of proximate analysis)

(%)	12% CP	16% CP	20% CP
Energy(kcal/kg)	2802.69	2837.01	2825.57
Protein	12.1166	15.9946	20.0966
Lysine	0.4761	0.62115	0.787
Methionine	0.21425	0.24827	0.28453
Calcium	1.3673	1.38775	1.41108
Phosphorous	0.37598	0.38403	0.3988
Fibre	3.6095	3.8105	4.2195
Ether Extract	3.7995	4.0495	4.2835

Table: 4.2 Preliminary results of semen parameters before treatment

Parameters	Group 1(12% CP) n* = 5	Group 2(16% CP) n = 5	Group 3(20% CP) n = 5
Live weight (kg)	4.1000 $\pm$ 0.2363	4.425 $\pm$ 0.1828	4.315 $\pm$ 0.2317
Semen Volume (mLs)	0.118 $\pm$ 0.008	0.123 $\pm$ 0.009	0.118 $\pm$ 0.008
pH	6.880 $\pm$ 0.372	7.083 $\pm$ 0.195	7.250 $\pm$ 0.204
Mass motility (%)	47.208 $\pm$ 2.390	52..500 $\pm$ 3.680	55.313 $\pm$ 3.996
Individual motility (%)	54.230 $\pm$ 3.731	59.480 $\pm$ 5.026	59.375 $\pm$ 2.997
Concentration ($\times 10^9$)	2.858 $\pm$ 0.499	3.530 $\pm$ 0.777	3.530 $\pm$ 0.862
% Live sperm (%)	55.500 $\pm$ 5.745	60.313 $\pm$ 5.163	61.250 $\pm$ 5.229
Total Defects (%)	35.43 $\pm$ 2.571	30.57 $\pm$ 1.852	32.49 $\pm$ 2.011

Table 4.3: Mean ($\pm$ SEM) semen parameters and live weights of turkey toms fed different levels of protein diets

Parameters	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
	n = 5	n = 5	n = 5
Live weight (kg)	5.29 $\pm$ 0.25	5.39 $\pm$ 0.20	5.63 $\pm$ 0.22
Semen Volume (mLs)	0.17 ^a $\pm$ 0.00	0.24 ^b $\pm$ 0.02	0.27 ^c $\pm$ 0.03
pH	6.86 $\pm$ 0.06	7.03 $\pm$ 0.06	6.85 $\pm$ 0.07
Mass motility (%)	73.01 ^a $\pm$ 1.34	79.09 ^b $\pm$ 1.36	80.00 ^b $\pm$ 1.19
Individual motility (%)	80.18 ^a $\pm$ 1.29	85.58 ^b $\pm$ 1.26	85.39 ^b $\pm$ 1.12
Semen Concentration ($\times 10^9$)	5.33 ^a $\pm$ 0.43	6.99 ^b $\pm$ 0.56	7.77 ^b $\pm$ 0.61
% Live sperm (%)	76.81 ^a $\pm$ 1.36	81.14 ^b $\pm$ 1.32	82.80 ^b $\pm$ 1.12
Total Defects (%)	18.43 $\pm$ 1.07	17.31 $\pm$ 0.99	16.89 $\pm$ 0.80

Values with different superscripts (across rows) differ significantly ($P < 0.005$)
n is the number of animals used.

4.3 Sperm Penetration Holes

The numbers of holes (mean $\pm$ SEM) of the *in vivo* and *in vitro* sperm penetration assay are presented in Table 4.4. Significant ($P < 0.05$) differences were observed between groups 1 and 2; and also between groups 1 and 3, but the results showed no significant ($P > 0.05$) difference between groups 2 and 3. The number of holes (mean $\pm$ SEM) of the *in vitro* sperm penetration assay are $148.88^a \pm 9.16$, $187.96^b \pm 8.121$ and $189.16^b \pm 6.46$ for groups 1, 2 and 3 respectively. The results revealed significant ($P < 0.05$) difference between groups 1 and 2; and also groups 1 and 3, but the difference between groups 2 and 3 was not significant.

Table 4.4: Mean number of sperm penetration holes for *in vivo* and *in vitro* assays using semen from turkey toms fed varying levels of protein diets.

Sperm Penetration Holes (/3 mm ²)			
	Group 1(12% CP)	Group 2 (16% CP)	Group 3 (20% CP)
No. of Animals	(n=5)	(n=5)	(n=5)
<i>In vivo</i>	136.80 ^a ± 9.28	160.97 ^b ± 8.08	172.83 ^b ± 7.65
<i>In vitro</i>	148.88 ^a ± 9.16	187.96 ^b ± 8.12	189.16 ^b ± 6.45

Values with different superscripts (across rows) differ significantly (P < 0.005)

4.4 Gonadal and Extra Gonadal Sperm/Spermatid Reserves

The mean ($\pm$ SEM) sperm reserves of testis, epididymis and *vas deferens* of the three groups of turkey, fed varying levels of protein diets are presented in Table 4.5.

The testicular sperm reserves per gram of testicle are $0.19^a \pm 0.00$, $0.21^b \pm 0.00$ and $0.21^b \pm 0.00$ for Groups 1, 2 and 3, respectively. There were significant ($P < 0.05$) differences in testicular sperm reserves between groups 1 and 2, and groups 1 and 3 ($P < 0.05$) but no significant ($P > 0.05$) difference between groups 2 and 3.

The mean ($\pm$ SEM) epididymis sperm reserves of the groups 1, 2 and 3 were $0.08^a \pm 0.00$, $0.12^b \pm 0.01$ and $0.18^c \pm 0.00$ respectively. There was significant ($P < 0.05$) difference between the testicular sperm reserves in groups 1 and 2, and between groups 1 and 3 ($P < 0.05$) but no significant ($P > 0.05$) difference was between groups 2 and 3.

The mean ($\pm$ SEM) *vas deferens* sperm reserves are 2.00 ± 0.06 , 2.82 ± 0.22 and 3.75 ± 0.27 . There was significant ($P < 0.05$) difference between the testicular sperm reserves in Groups 1 and 2, and Groups 1 and 3, but difference between groups 2 and 3 was not significant.

Table 4.5: Gonadal and Extra gonadal sperm reserves of turkey toms fed varying levels of protein diets

Gonadal and Extra gonadal sperm reserves (paired organs)			
	Group 1(12% CP)	Group 2 (16% CP)	Group 3 (20% CP)
No of Animals	(n=3)	(n=3)	(n=3)
Testicles sperm reserve ($\times 10^9/\text{gm}$)	$0.19^a \pm 0.00$	$0.21^b \pm 0.00$	$0.21^b \pm 0.00$
Epididymis sperm reserve ($\times 10^9$)	$0.08^a \pm 0.00$	$0.12^b \pm 0.01$	$0.18^c \pm 0.00$
<i>Vas deferens</i> sperm reserve ($\times 10^9$)	$2.00^a \pm 0.06$	$2.82^b \pm 0.22$	$3.75^c \pm 0.27$
Values with different superscripts (across rows) differ significantly ($P < 0.005$)			

Figure 4.1: The weekly trend in live weights of turkey toms fed varying levels of protein diets.

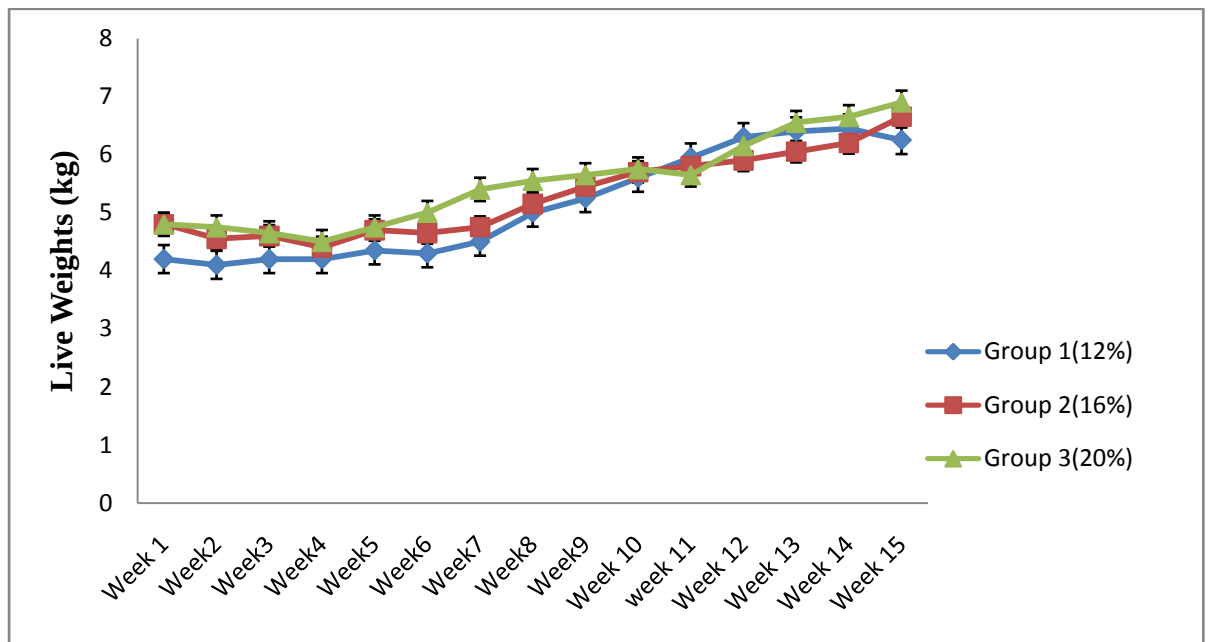


Figure 4.2: The weekly trend in semen volumes of turkey toms fed varying levels of protein diets.

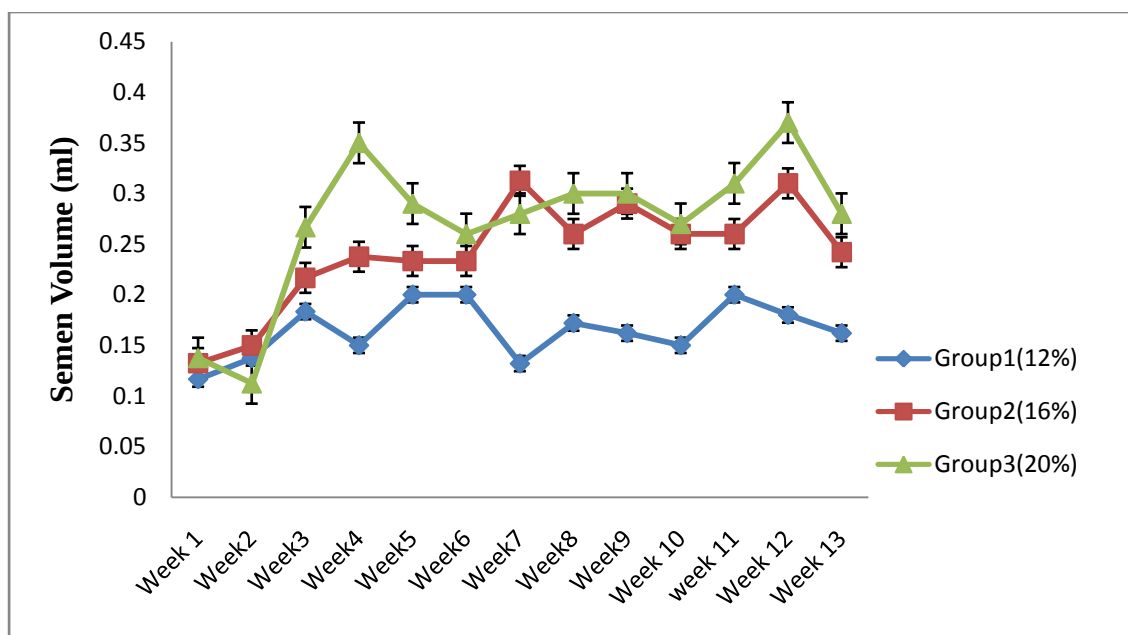
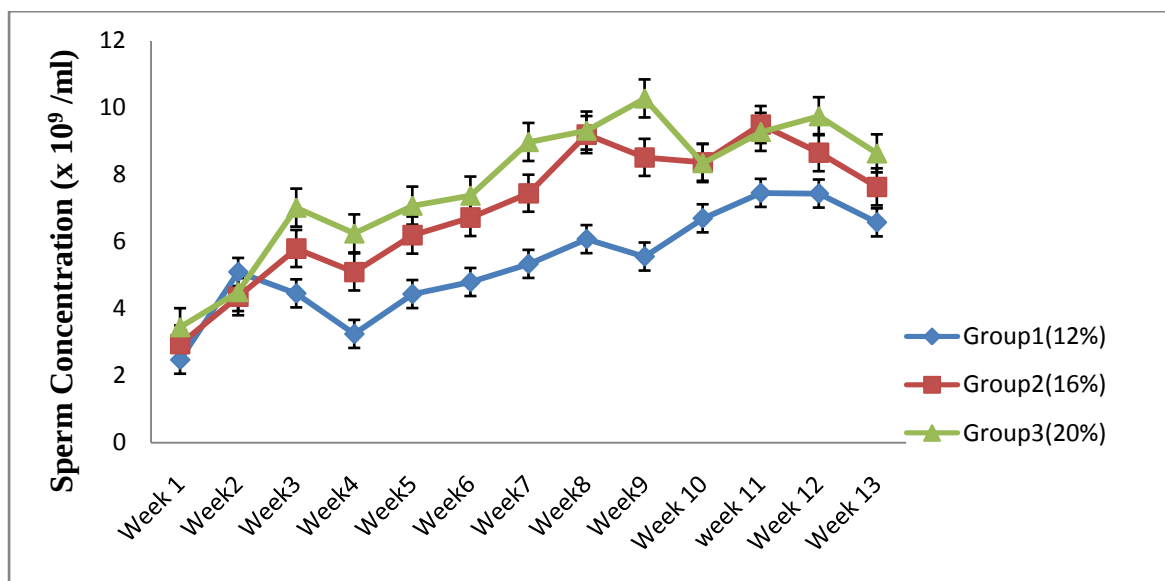


Figure 4.3: The weekly trend in semen concentration of turkey toms fed varying levels of protein diets.



4.5 Correlation Matrix between Live Weight and Semen Parameter of Turkey Toms Fed Varying Levels of Protein Diets.

The results of correlation analysis between live weights and semen parameters; that is, semen volume, concentration, number of percentage live sperm cells and percentage normal spermatozoa, in all the three groups are shown in Table 4.4. Group 1 had $r = (0.28, 0.90, 0.64 \text{ and } 0.51)$, for semen volume, concentration, live sperm count and normal sperm count, respectively. In groups 2 and 3 however, the values are $r = (0.67, 0.83, 0.55 \text{ and } 0.60)$ and $r = (0.61, 0.78, 0.65 \text{ and } 0.50)$, respectively. There was positive correlation between live weights and most of the semen parameters in all the groups and there was significant difference between the correlation coefficients of the groups.

Table 4.6: Correlation matrix between live weight and semen parameters of turkey toms fed varying levels of protein diets.

Semen parameters	Live Weights		
	Group 1 (12%) CP	Group 2 (16%) CP	Group 3 (20%) CP
	(r)	(r)	(r)
Volume	0.28 ^a	0.67 ^b	0.61 ^c
Concentration	0.90 ^a	0.83 ^b	0.78 ^c
% Live	0.64 ^a	0.55 ^b	0.65 ^a
% Normal	0.51 ^a	0.60 ^b	0.50 ^a

Values with different superscripts (across rows) differ significantly ($P < 0.005$)

4.6 Haemogram

Mean ($\pm$ SEM) values of blood parameters are presented in table 4.7. The mean Packed Cell Volume (PCV) and Haemoglobin Concentration (Hb) values revealed significant differences ($P < 0.05$) between groups 1 and 2 and groups 1 and 3, but there was no significant difference ($P > 0.05$) between groups 2 and 3. The mean WBC counts show significant differences between all the groups while no significant differences were found between all the groups in the mean values of total protein, heterophils, lymphocytes, monocytes eosinophils, basophils and band cells.

Table 4.7: Mean values of blood parameters of turkey toms fed varying Levels of protein diets.

Blood parameters			Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
Packed Cell Volume (%)			29.20 ± 2.85 ^a	33.8 ± 1.39 ^b	32.6 ± 1.54 ^b
White Blood Cell			3.32 ± 0.68 ^a	6.08 ± 2.37 ^b	3.80 ± 0.51 ^a
(X10 ³ /µl)					
Heterophils (%)			9.40 ± 1.03	7.60 ± 2.25	8.80 ± 1.74
Lymphocytes (%)			90.40 ± 1.17	91.60 ± 2.50	88.80 ± 0.97
Monocytes (%)			1	2	3
Eosinophils (%)			0	1	3
Basophils (%)			0	0	0
Band Cells (%)			0	0	0
Total Protein			4.60 ± 0.44	4.24 ± 0.50	4.32 ± 0.27
Haemoglobin (g/dL)			7.90 ± 1.90 ^a	11.24 ± 0.47 ^b	10.84 ± 0.51 ^b
Values with different superscripts (across rows) differ significantly (P < 0.005)					

CHAPTER FIVE

DISCUSSION

Live weights of the turkey toms taken throughout the period of the study showed increase in the weights of the toms in all the groups which were not dramatic and there were no significant differences ($P > 0.05$) between the mean ($\pm$ SEM) live weights of the three groups. This was perhaps partly because the feed was restricted to 400g/tom/day and partly because the feeds were isocaloric, the differences were therefore not expected to be very significant.

The results of the different values of semen parameters of toms fed 12%, 16% and 20% CP showed that semen volume was higher in group 3 (20% CP), followed by group 2 (16% CP) with Group 1 (12% CP) having the least volume. There was no significant ($P < 0.05$) difference between Group 2 (16% CP) and group 3 (20% CP). The significant differences between group 1 and group 2, and between group 1 and Group 3, were probably due to the different protein levels fed. This is contrary to the reports of Perry (1960) and Etches (1996) in cockerels and Jibril *et al.* (2011) in rams, who reported higher reproductive performance in lower CP level than in higher CP levels. The results are in agreement with those reported by Rekwot *et al.*, (1987), and Rekwot *et al.*, (1988) in bulls, Louis *et al.* (1994) in boars, Ladokun *et al.* (2006) in pubertal rabbit bucks, Ghonim *et al.* (2010) in drakes, who reported better performance in animals fed higher CP levels than those fed lower CP levels. It also agrees with the work of Sotirov *et al.* (2002) who reported higher ejaculate volume in turkeys fed 17% CP than in their counterparts fed 14% CP.

Although the ejaculate volumes obtained in Group 1 (12% CP) were similar to those reported by Zahraddeen *et al.* (2005) in indigenous turkey toms, they were not within the ranges reported by Burke (1984), Bakst (1990) and Christensen (2005) in exotic

breeds. The volumes obtained in group 2 (16% CP) and group 3 (20% CP) were within the range given for the exotic breeds and was higher than the 0.17 ± 0.02 mls reported by Zahraddeen *et al.* (2005) in local breeds, but similar to the report by Yahaya *et al.* (2013). The differences observed between the values in this work and the values reported by Zahraddeen *et al.* (2005) in the same breed might be due to the difference in feeding. Zahraddeen *et al.* (2005) fed 10% CP which was lower than the 12%, 16% and 20% used in the present study.

Semen concentration was significantly higher in group 3 than in Group 1, the result in group 2 was also significantly higher than that in group 1. This was apparently due to the effect of the difference in the levels of protein in their diets. This disagrees with the works by Jibril *et al.* (2011) who reported higher semen concentration in a group of rams fed 15% CP than that of their counterparts fed 18% CP. This might be attributed to the optimum utilization of dietary protein at about 15% CP in that specie and breed. The result, however, is similar to that reported by Rekwot *et al.* (1987) and Rekwot *et al.* (1988) in bulls; Louis *et al.* (1994) in boars; Ladokun *et al.* (2006) in pubertal rabbit bucks; Ghonim *et al.* (2010) in drakes, who reported better performance in animals fed higher CP levels than those fed lower CP levels. It also agrees with the report of Sotirov *et al.* (2002), who observed higher semen concentration in turkeys fed 17% CP than in those fed 14% CP. There was however no significant difference between group 2 and Group 3. This might be attributed to the optimum utilization of dietary protein at about 16-20% CP in this breed of turkeys, considering the results obtained in the present study. Semen concentration in the present study is higher than the $2.8 \pm 74.3 \times 10^9$ /ml and $4.66 \pm 70.73 \times 10^9$ /ml reported by Zahraddeen *et al.* (2005) in local and exotic breeds, respectively. The values obtained in Groups 2 and 3 are, however, similar to the report by Yahaya *et al.* (2013)

($6.22 \pm 0.245 \times 10^9$ /ml) and that by Cecil and Bakst (1988) ($7.90 \pm 1.19 \times 10^9$ /ml). They also fell within the range of 6-12 billions per ml reported by Christensen (2005) for exotic breeds.

The significant differences observed between groups 1 and 2 and also between groups 1 and 3 may be attributed to the difference in the level of crude protein in their diets. This agreed with the work by Sotirov *et al.* (2002), who observed increase in both mass and individual motilities of sperm in turkeys fed 17% CP than in those fed 14% CP. It also agreed with the report by Ghonim *et al.* (2010), who reported higher sperm motility though without significant difference in drakes fed higher crude protein than in those fed lower crude protein.

There was no significant difference ($P < 0.05$) between groups 2 and 3 in terms of live sperm count. There was, however, significant difference between the two groups and group 1, this is perhaps so because optimum protein level increased the viability of the sperm cells in groups 2 and 3 as against group 1, where 12% CP which was fed may be considered suboptimal in this breed. The result disagreed with that by Jibril *et al.* (2011), who observed no difference in sperm viability of Yankasa rams placed on different protein diets. However it agreed with that by Sotirov *et al.*, (2002) and Ghonim *et al.* (2010) who observed significant differences in the percentage live sperm of turkeys and drakes respectively fed varying levels of crude protein.

The difference observed in the values of sperm morphology count between all the groups was not significant ($P < 0.05$). This agreed with the result of Sotirov *et al.* (2002) and Jibril *et al.* (2011), who reported no significant difference in the percentage of abnormal sperm in turkeys and rams, respectively, fed varying levels of protein diet. The result disagreed with that by Ghonim *et al.* (2010), who found differences in drakes, based on differences in the level of protein consumed.

There was significant difference between groups 1 and 3 and also between groups 1 and 2 but there was no significant difference between groups 2 and 3 in the number of both the *in vivo* and *in vitro* sperm penetration holes. The significant differences observed between groups 1 and 2 and also between groups 1 and 3 may be attributed to the difference in the level of crude protein in their diets. The results of the *in vitro* sperm penetration assay are lower than that by Barbato *et al.* (1998) who observed up to 800 /mm² sperm penetration holes tested at 5×10^6 sperm cells per tube using fresh semen. This difference might be attributed to the difference in equipments used between the two experiments. The result of the *in vivo* sperm penetration assay was similar to the report by Wishart and Staines (1998), who reported means of 6.6, 42.9 and 62.3 in hens, inseminated with sperm from roosters with increasing dietary energy content. The result of this work indicates that all the three groups had the efficiency to fertilize eggs, but group 3 was the most efficient followed by group 2 and then group 1. This findings suggests that increased protein level enhances the fertilizing ability of semen.

Significant difference ($P < 0.05$) was observed in all the parameters of testicular, epididymal and *vas deferens* reserves between the Groups. This might be attributed to the variation in the crude protein contents of their various diets. This result agrees with the work of Ladokun *et al.* (2006), who observed significant difference in gonadal and extragonadal sperm reserves in rabbit bucks fed varying protein diets. The reserves are similar to the result of Cecil *et al.* (1988), who reported $122-127 \times 10^6$ sperm cells/g, 58×10^6 (ejaculated) and 204×10^6 sperm cells (rested), 3160×10^6 sperm cells (ejaculated) and 10320×10^6 sperm cells (rested), and 3248×10^6 sperm cells (ejaculated) and 10524×10^6 sperm cells (rested) for gonadal sperm reserve, epididymal, *vas deferens* and total extragonadal reserves, respectively.

Figures 2 and 3 show the trends in ejaculate volume and semen concentration of the three groups. Although all the groups had a steady rise in the values of the two parameters throughout the weeks, the increase was more in Group 3 followed by Group 2 and then Group 1. This variability was, apparently, due to the variation in the crude protein content of the diet fed.

There is positive correlation ($P < 0.05$) between live weights of the toms and their semen parameter such as semen volume, semen concentration, live sperm and percentage normal cells. Although the difference in the correlation coefficient between the three groups is not significant, it can be seen that the live weight is poorly correlated to volume in Group 1, while it is positively correlated in Group 2 and 3. This result agreed with the finding of Barth and Chaudhari (2002) in cocks, Butswat and Zaharadden (1998) in bucks. The reason for the observed correlation may be because the optimum protein utilization in turkeys is between 16% CP and 22% CP. The observed difference between groups 2 and 3 and group 1 may be as a result of the difference in protein content of their diet, while 12% CP is perhaps more far away from the optimum, 16% CP and 20% CP are closer to it; and, hence, the similar values in the correlation matrix.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION

6.0 Summary

Preliminary results, pretreatment, showed no significant difference in the semen characteristics between groups 1, 2 and 3.

During treatment with 12% CP, 16% CP and 20% CP for groups 1, 2 and 3, respectively, significant difference was found in the semen characteristics and sperm fertilizing ability between the groups.

Groups 2 and 3 had better semen quality as seen in the semen characteristics than group 1.

After treatment, gonadal and extra gonadal sperm reserves analysis showed better results for groups 2 and 3 Compared to group 1.

6.1 Conclusions

From the results of this study it is concluded that

1. Dietary protein levels influenced semen production of the indigenous turkey toms as shown by increased semen volume, increased semen concentration sperm viability gonadal and extra gonadal sperm reserves with increased dietary protein levels.
2. The protein in the diet influenced the fertilizing ability of the toms' spermatozoa as shown by variation in the number of *in vivo* and *in vitro* sperm penetration holes observed on the perivitelline membrane (PVM) of eggs fertilized by toms fed varying protein levels.

6.2 Recommendations

The following recommendations were made;

Artificial Insemination (AI), in the indigenous Nigerian turkey breeds, using fresh semen should be encouraged among the local turkey farmers.

Crude protein levels of 16-20% should be adopted pending when the optimal level is ascertained by a more extensive research.

Sperm penetration assays should be employed in the indigenous turkey toms for the detection of superior males in terms of fertility.

Further work should be carried out to determine the optimal protein and energy levels for indigenous Nigerian turkey toms.

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APPENDICES

Appendix I

Average weekly semen volumes collected from turkey toms fed varying levels of protein diet

	Grp1(12% CP)	Grp2(16% CP)	Grp3(20% CP)
Week 1	0.12	0.13	0.14
Week2	0.14	0.15	0.11
Week3	0.18 ^a	0.22 ^b	0.27 ^b
Week4	0.15 ^a	0.24 ^b	0.35 ^c
Week5	0.20 ^a	0.23 ^a	0.29 ^b
Week6	0.20	0.23	0.26
Week7	0.13 ^a	0.31 ^b	0.28 ^b
Week8	0.17 ^a	0.26 ^b	0.30 ^c
Week9	0.16 ^a	0.29 ^b	0.30 ^b
Week 10	0.15 ^a	0.26 ^b	0.27 ^b
week 11	0.20 ^a	0.26 ^b	0.31 ^c
Week 12	0.18 ^a	0.31 ^b	0.37 ^b
Week 13	0.16 ^a	0.24 ^b	0.28 ^b
Mean ± SEM	0.17 ^a ± 0.01	0.25 ^b ± 0.01	0.27 ^b ± 0.02

Values with different superscripts (across rows) differ significantly ($P < 0.005$).

Appendix II

Average weekly semen concentrations of turkey toms fed varying levels of protein diet.

	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
Week 1	2.30	2.55	2.85
Week2	4.83	4.93	4.63
Week3	4.46 ^a	5.80 ^b	7.02 ^c
Week4	3.25 ^a	5.10 ^b	6.24 ^c
Week5	4.44 ^a	6.20 ^b	7.08 ^c
Week6	4.80 ^a	6.73 ^b	7.38 ^b
Week7	5.34 ^b	7.45 ^b	8.98 ^c
Week8	6.08 ^a	9.20 ^b	9.32 ^b
Week9	5.56 ^a	8.52 ^b	10.28 ^c
Week 10	6.70 ^a	8.38 ^b	8.35 ^b
week 11	7.46 ^a	9.50 ^b	9.28 ^b
Week 12	7.44 ^a	8.66 ^b	9.75 ^c
Week 13	6.58 ^a	7.88 ^b	9.80 ^c
Means (± SEM)	5.33 ^a ± 0.43	6.99 ^b ± 0.56	7.77 ^b ± 0.61

Values with different superscripts (across rows) differ significantly (P < 0.005).

Appendix III

Average weekly normal sperm cells' count of turkey toms fed varying levels of protein diet.

	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
Week 1	77.60	70.50	78.25
Week2	71.25	73.66	70.50
Week3	79.60	89.75	86.00
Week4	79.25	79.00	80.40
Week5	74.40	73.75	74.80
Week6	81.50	79.20	80.00
Week7	79.80	75.00	83.00
Week8	92.00	84.00	88.40
Week9	89.20	89.40	92.20
Week 10	94.60	88.75	88.50
week 11	68.40	82.25	80.20
Week 12	87.60	92.40	92.25
Week 13	87.80	83.60	83.00
Means ($\pm$ SEM)	81.575 $\pm$ 1.068	82.692 $\pm$ 0.996	83.112 $\pm$ 0.804

Appendix IV

Average weekly mass motility count of spermatozoa from turkey toms fed varying levels of protein diets.

	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
Week 1	44.00	45.00	45.00
Week2	52.50	50.00	53.75
Week3	62.00 ^a	68.75 ^a	82.00 ^b
Week4	81.25	82.50	79.00
Week5	78.00 ^a	90.00 ^b	84.00 ^c
Week6	80.00	90.00	91.00
Week7	84.00	81.25	87.00
Week8	66.00 ^a	83.00 ^b	75.00 ^b
Week9	79.00	84.00	85.00
Week 10	87.00	83.75	85.00
week 11	80.00	76.25	78.00
Week 12	83.00	84.00	92.50
Week 13	85.00	84.00	86.00
Means ($\pm$ SEM)	73.01 ^a $\pm$ 1.34	79.09 ^b $\pm$ 1.361	80.00 ^b $\pm$ 1.19

Values with different superscripts (across rows) differ significantly ($P < 0.005$).

Appendix V

Average weekly individual motility count of spermatozoa from turkey toms fed varying levels of protein diets.

	Individual Motility (%)		
	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
Week 1	50.00 ^a	60.00 ^b	52.50 ^a
Week2	61.25 ^a	46.66 ^b	65.00 ^a
Week3	69.00 ^a	82.50 ^b	87.00 ^b
Week4	85.00	90.00	81.00
Week5	82.00	90.00	84.00
Week6	87.50 ^a	92.50 ^b	93.00 ^b
Week7	89.00	91.25	92.00
Week8	77.00	88.00	87.00
Week9	86.00	94.00	92.00
Week 10	92.00	91.25	90.00
week 11	90.00	86.25	88.00
Week 12	88.00 ^a	92.00 ^b	95.00 ^b
Week 13	91.00	90.00	91.00
Means (±SEM)	80.177 ^a ± 1.288	85.577 ^b ± 1.262	85.388 ^b ± 1.119

Values with different superscripts (across rows) differ significantly (P < 0.005).

Appendix VI

Average weekly percentage of dead cells' count of spermatozoa from turkey toms fed varying levels of protein diets.

Dead cells (%)			
	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
Week 1	58.00	55.00	52.50
Week2	36.25	35.00	31.25
Week3	34.00	32.50	30.00
Week4	20.00	15.00	18.00
Week5	20.00	11.25	15.00
Week6	17.50	6.25	9.00
Week7	29.00	26.25	15.00
Week8	18.00	29.00	10.00
Week9	11.00	13.00	13.00
Week 10	17.00	13.75	10.00
week 11	22.00	16.25	23.00
Week 12	18.00	10.00	8.75
Week 13	12.00	8.00	11.00
Means ($\pm$ SEM)	23.19 ^a $\pm$ 1.37	18.87 ^b $\pm$ 1.32	17.20 ^b $\pm$ 1.12

Values with different superscripts (across the rows) differ significantly ($P < 0.005$).

Appendix VII

Average weekly percentage of live cells' count of spermatozoa from turkey toms fed varying levels of protein diets.

Live Cells (%)			
	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
Week 1	42.00	45.00	47.50
Week2	63.75	65.00	68.75
Week3	66.00	67.50	70.00
Week4	80.00	85.00	82.00
Week5	80.00	88.75	85.00
Week6	82.50	93.75	91.00
Week7	71.00	73.75	85.00
Week8	82.00	71.00	90.00
Week9	89.00	87.00	87.00
Week 10	83.00	86.25	90.00
week 11	78.00	83.75	77.00
Week 12	82.00	90.00	91.25
Week 13	88.00	92.00	89.00
Means ($\pm$ SEM)	76.81 ^a $\pm$ 1.36	81.14 ^b $\pm$ 1.32	82.802 ^b $\pm$ 1.12

Values with different superscripts (across the rows) differ significantly ($P < 0.005$).

Appendix VIII

Mean weekly pH of semen from turkey toms fed varying levels of protein diets.

	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
Week 1	6.40	6.75	6.75
Week2	7.75	7.33	7.25
Week3	6.40	7.00	6.20
Week4	7.00	7.50	7.00
Week5	7.40	7.50	6.80
Week6	6.75	7.25	7.20
Week7	6.40	6.75	6.60
Week8	6.60	7.00	6.60
Week9	7.00	7.00	6.80
Week 10	7.00	6.75	6.75
week 11	6.80	7.00	6.80
Week 12	7.00	7.00	6.75
Week 13	7.00	7.00	7.00
Means ($\pm$ SEM)	6.86 $\pm$ 0.06	7.03 $\pm$ 0.06	6.85 $\pm$ 0.07

Values with different superscripts (across the rows) differ significantly ($P < 0.005$).

Appendix IX

Mean weekly reaction times of turkey toms fed varying levels of protein diets.

	Reaction Time (seconds)		
	Group 1(12% CP)	Group 2(16%CP)	Group 3(20% CP)
Week 1	31.00 ^a	13.80 ^b	31.00 ^a
Week2	9.00	9.00	9.00
Week3	6.20 ^a	4.00 ^b	3.60 ^b
Week4	14.50 ^a	6.00 ^b	4.80 ^c
Week5	4.80 ^a	5.60 ^b	4.00 ^c
Week6	5.20 ^a	3.00 ^b	3.20 ^b
Week7	6.20 ^a	5.40 ^b	4.80 ^c
Week8	5.40 ^a	4.20 ^b	4.60 ^b
Week9	2.20	2.60	2.40
Week 10	2.60	3.80	3.20
week 11	2.40	2.80	2.60
Week 12	2.20	2.80	2.40
Week 13	2.40	2.20	3.00
Means ($\pm$ SEM)	6.70 ^a $\pm$ 0.87	4.20 ^b $\pm$ 0.26	4.66 ^b $\pm$ 0.42

Values with different superscripts (across the rows) differ significantly ($P < 0.005$).

Appendix X

Mean weekly abnormal cells' count of spermatozoa from turkey toms fed 12% CP diet.

	Abnormal Cell count (%)			
	Group1 (12% CP)			
	MC	CT	BH	FH
Week 1	4.80	4.20	11.00	2.80
Week2	12.25	5.75	5.00	5.75
Week3	5.80	6.00	3.80	5.2
Week4	4.00	3.75	10.75	2.75
Week5	8.00	5.60	7.00	5.4
Week6	6.50	4.75	5.50	1.25
Week7	5.40	5.60	4.40	4.8
Week8	1.60	2.00	3.00	1.40
Week9	1.00	3.20	0.40	6.20
Week 10	0.60	1.00	0.60	3.20
week 11	8.20	12.00	5.00	6.60
Week 12	5.20	3.60	1.00	2.60
Week 13	0.80	0.80	3.40	7.40
Mean $\pm$ SEM	4.94 $\pm$ 0.95	4.48 $\pm$ 0.79	4.68 $\pm$ 0.94	4.26 $\pm$ 0.56

Appendix XI

Mean weekly abnormal cells' count of spermatozoa from turkey toms fed 16% CP diet.

Abnormal Cell count (%)				
Group 2 (16% CP)				
	MC	CT	BH	FH
Week 1	10.00	9.00	8.25	2.25
Week2	6.33	7.33	10.00	3.00
Week3	3.75	2.00	1.50	3.00
Week4	7.00	7.50	5.00	1.50
Week5	9.00	6.00	5.75	5.50
Week6	7.80	5.40	7.20	1.40
Week7	7.25	10.50	6.00	1.25
Week8	4.60	3.00	3.60	4.60
Week9	0.60	3.60	2.00	2.80
Week 10	1.75	4.75	3.00	1.75
week 11	6.50	4.75	4.25	1.75
Week 12	1.80	2.00	1.80	2.00
Week 13	2.80	6.80	5.80	1.00
Mean $\pm$ SEM	5.32 $\pm$ 0.83	5.59 $\pm$ 0.72	4.94 $\pm$ 0.72	2.45 $\pm$ 0.37

Appendix XII

Mean weekly abnormal cells' count of spermatozoa from turkey toms fed 20% CP diet.

Abnormal Cell count (%)				
Group 3 (20% CP)				
	MC	CT	BH	FH
Week 1	5.25	4.75	9.50	2.75
Week2	10.00	9.00	8.25	2.25
Week3	3.00	4.00	3.40	3.60
Week4	5.20	6.40	5.60	2.40
Week5	5.80	7.60	8.40	3.80
Week6	7.60	4.40	4.80	1.20
Week7	5.80	5.00	5.80	1.00
Week8	2.80	2.40	4.60	1.80
Week9	1.00	1.80	1.20	3.80
Week 10	2.25	2.25	4.25	2.75
week 11	4.00	5.80	5.00	5.20
Week 12	2.75	0.75	1.75	3.25
Week 13	3.20	5.60	4.60	4.00
Means (± SEM)	4.51 ± 0.68	4.596 ± 0.66	5.165 ± 0.68	2.90 ± 0.33

Appendix XIII

Mean weekly sperm penetration holes count using semen from turkey toms (individuals) fed varying Levels of protein diets. (At the dose of 5million spermatozoa per tube)

	Individuals Sperm Penetration Holes (/3mm ²)		
	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
Week 1	148.00 ^a	192.60 ^b	161.80 ^c
Week2	134.80 ^a	182.40 ^b	205.20 ^c
Week3	171.80 ^a	177.20 ^a	201.40 ^b
Week4	152.00 ^a	183.20 ^b	189.60 ^b
Week5	137.80 ^a	204.40 ^b	187.80 ^c
Means (± SEM)	148.88 ^a ± 9.16	187.96 ^b ± 8.12	189.16 ^b ±6.45

Values with different superscripts (across the rows) differ significantly (P < 0.005).

Appendix XIV

Mean weekly sperm penetration holes count using semen from turkey toms (groups) fed varying Levels of protein diets. (At the dose of 5 million spermatozoa per tube)

Groups Sperm Penetration Holes (/3mm ²)			
	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
Week 1	157.00 ^a	184.67 ^b	195.00 ^c
Week2	186.00 ^a	169.33 ^b	152.33 ^c
Week3	92.33 ^a	107.33 ^b	150.67 ^c
Week4	125.33 ^a	156.00 ^b	176.00 ^c
Week5	86.00 ^a	142.33 ^b	161.00 ^c
Week6	114.00 ^a	157.33 ^b	183.33 ^c
Week7	133.33 ^a	168.00 ^b	185.67 ^c
Week8	163.67 ^a	135.00 ^b	133.67 ^b
Means (± SEM)	136.80 ^a ± 9.28	160.97 ^b ± 8.08	172.83 ^b ± 7.65
Values with different superscripts (across the rows) differ significantly (P < 0.005).			

Appendix XV

Mean testicular circumferences of turkey toms fed varying Levels of protein diets

Testicular Circumferences (cm)				
Turkey toms	Organs	Group1 (12%CP)	Group 2(16% CP)	Group 3(20% CP)
B	Left	8.00	6.50	9.30
	Right	5.00	5.60	6.90
C	Left	6.00	6.40	6.00
	Right	5.50	5.70	6.00
D	Left	6.50	7.20	5.30
	Right	6.00	6.80	6.10
Mean ± SEM		6.17± 0.42	6.37 ± 0.25	6.600 ± 0.58

Appendix XVI

Mean testicular lengths of turkey toms fed varying Levels of protein diets

Testicular Lengths (cm)				
Toms	Organs	Group 1(12% CP)	Group 2(16% CP)	Group 3(20%CP)
B	Left	4.00	4.00	7.00
	Right	3.10	3.80	3.70
C	Left	4.00	5.00	4.00
	Right	3.50	4.20	4.00
D	Left	4.00	5.00	4.20
	Right	3.70	4.40	4.00
Mean $\pm$ SEM		3.72 ^a $\pm$ 0.15	4.40 ^b $\pm$ 0.21	4.48 ^b $\pm$ 0.51

Values with different superscripts (across the rows) differ significantly ($P < 0.005$).

Appendix XVII

Mean *Vas deferens* lengths of turkey toms fed varying Levels of protein diets

VD Lengths (cm)				
Toms	Organs	Group 1(12%CP)	Group 2(16%CP)	Group 3(20% CP)
B	Left	20	17	18
	Right	19	16	18
C	Left	17.5	18	18.8
	Right	16	17	15.8
D	Left	16	19	17
	Right	17	17	16
Mean $\pm$ SEM		17.583 $\pm$ 0.67	17.33 $\pm$ 0.42	17.27 $\pm$ 0.49

Appendix XVIII

Mean epididymal length of turkey toms fed varying Levels of protein diets

Epididymal Lengths (cm)				
Toms	Organs	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
B	Left	4.00 ^a	3.30 ^b	3.00 ^b
	Right	3.30 ^a	2.50 ^b	2.00 ^c
C	Left	3.80 ^a	2.30 ^b	3.40 ^a
	Right	2.70 ^a	4.00 ^b	2.90 ^a
D	Left	4.00 ^a	3.40 ^b	3.50 ^b
	Right	3.10 ^a	3.20 ^a	4.20 ^b
Mean ± SEM		3.48 ^a ± 0.22	3.12 ^b ± 0.25	3.17 ^b ± 0.30

Values with different superscripts (across the rows) differ significantly ($P < 0.005$).

Appendix XIX

Mean testicular weights of turkey toms fed varying Levels of protein diets

Testicular Weights (g)				
Toms	Organs	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
B	Left	13.40 ^a	9.20 ^b	16.50 ^c
	Right	6.60 ^a	8.40 ^b	9.90 ^c
C	Left	7.10 ^a	8.40 ^b	8.80 ^b
	Right	6.60 ^a	7.20 ^b	8.40 ^c
D	Left	8.10 ^a	9.10 ^b	7.90 ^a
	Right	7.80 ^a	8.70 ^b	7.50 ^a
Mean ± SEM		8.27 ^a ± 1.06	8.50 ^b ± 0.29	9.83 ^c ± 1.38

Values with different superscripts (across the rows) differ significantly (P < 0.005).

Appendix XX

Mean epididymal weights of turkey toms fed varying Levels of protein diets

Epididymal Weights (g)				
Toms	Organs	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
B	Left	0.70 ^a	0.40 ^b	0.40 ^b
	Right	0.40 ^a	0.30 ^b	0.20 ^c
C	Left	0.30 ^a	0.24 ^b	0.32 ^a
	Right	0.30 ^a	0.50 ^b	0.31 ^a
D	Left	0.30 ^a	0.37 ^b	0.22 ^c
	Right	0.40 ^a	0.17 ^b	0.21 ^c
Mean ± SEM		0.40 ^a ± 0.06	0.3300 ^b ± 0.05	0.28 ^c ± 0.03

Values with different superscripts (across the rows) differ significantly (P < 0.005).

Appendix XXI

Mean *Vas deferens* weight of turkey toms fed varying Levels of protein diets.

VD Weights (g)				
Toms	Organs	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
B	Left	0.60 ^a	0.50 ^b	0.40 ^c
	Right	0.40 ^a	0.30 ^b	0.30 ^b
C	Left	0.50 ^a	0.54 ^a	0.44 ^b
	Right	0.30 ^a	0.24 ^b	0.25 ^b
D	Left	0.30 ^a	0.74 ^b	0.46 ^c
	Right	0.30 ^a	0.68 ^b	0.33 ^a
Mean ± SEM		0.40 ^a ± 0.05	0.50 ^b ± 0.08	0.36 ^c ± 0.03

Values with different superscripts (across the rows) differ significantly (P < 0.005).

Appendix XXII

Mean testicular sperm reserves of turkey toms fed varying Levels of protein diets.

Testicular Reserve/gram (x10⁶)

Toms	Organs	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
B	Left	175 ^a	218 ^b	210 ^b
	Right	180 ^a	206 ^b	205 ^b
C	Left	184 ^a	204 ^b	216 ^b
	Right	180 ^a	202 ^b	210 ^b
D	Left	196 ^a	209 ^b	222 ^b
	Right	195 ^a	199 ^a	207 ^b
Mean ± SEM		185.00 ^a ± 3.52	206.33 ^b ± 2.72	211.67 ^b ± 2.57

Values with different superscripts (across the rows) differ significantly (P < 0.005).

Appendix XXIII

Mean epididymal sperm reserves of turkey toms fed varying Levels of protein diets.

Epididymal Reserves (x10 ⁶)				
Toms	Organs	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
B	Left	70 ^a	106 ^b	126 ^c
	Right	68 ^a	102 ^b	119 ^c
C	Left	85 ^a	123 ^b	201 ^c
	Right	80 ^a	89 ^a	197 ^b
D	Left	88 ^a	156 ^b	202 ^c
	Right	82 ^a	113 ^b	205 ^c
Mean ± SEM		78.83 ^a ± 3.31	114.83 ^b ± 9.44	175.00 ^c ± 16.66

Values with different superscripts (across the rows) differ significantly (P < 0.005).

Appendix XXIV

Mean *Vas deferens* sperm reserves of turkey toms fed varying Levels of protein diets.

VD Reserves (x10 ⁹)				
Toms	Organs	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
B	Left	2.00 ^a	1.90 ^b	4.30 ^c
	Right	1.90 ^a	2.60 ^b	3.60 ^c
C	Left	1.80 ^a	3.30 ^b	3.20 ^b
	Right	2.10 ^a	2.90 ^b	2.90 ^b
D	Left	2.20 ^a	3.40 ^b	4.70 ^c
	Right	2.00 ^a	2.80 ^b	3.80 ^c
Mean ± SEM		2.00 ^a ± 0.06	2.817 ^b ± 0.22	3.75 ± 0.27 ^c

Values with different superscripts (across the rows) differ significantly (P < 0.005).

Appendix XXV

Mean *Vas deferens* diameters of turkey toms fed varying Levels of protein diets.

VD Diameter (cm)				
Toms	Organs	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
B	Left	0.20	0.22	0.20
	Right	0.19	0.19	0.25
C	Left	0.18	0.18	0.19
	Right	0.17	0.2	0.21
D	Left	0.20	0.23	0.24
	Right	0.19	0.20	0.23
Mean ± SEM		0.19 ± 0.00	0.20 ± 0.01	0.22 ± 0.01

Appendix XXVI

Mean weights of tunicae albuginea of turkey toms fed varying levels of protein diets.

Weights of Tunicae Albuginea (cm)

Toms	Organs	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
B	Left	0.67 ^a	0.46 ^b	0.83 ^c
	Right	0.33	0.42	0.50
C	Left	0.36	0.42	0.44
	Right	0.33 ^a	0.36 ^a	0.42 ^b
D	Left	0.40	0.46	0.40
	Right	0.39	0.44	0.38
Mean ± SEM		0.41 ^a ± 0.05	0.43 ^a ± 0.01	0.49 ^b ± 0.07

Values with different superscripts (across the rows) differ significantly (P < 0.005).

Appendix XXVII

Correlation Matrix between live weight and semen parameter of turkey toms fed 12% CP diet.

	Live Weight	Volume	Concentration	% Live	% Normal
Live Weight		0.28	0.90	0.64	0.52
Volume	0.28		0.40	0.55	0.10
Concentration	0.90	0.40		0.62	0.32
% Live cells	0.64	0.55	0.62		0.48
% Normal Cells	0.52	0.10	0.32	0.48	

Appendix XXVIII

Correlation Matrix between live weight and semen parameter of turkey toms fed 16% CP diet.

	Live Weight	Volume	Concentration	% Live	% Normal
Live Weight		0.67	0.83	0.55	0.60
Volume	0.67		0.83	0.66	0.59
Concentration	0.83	0.83		0.61	0.66
% Live Cells	0.55	0.66	0.61		0.46
% Normal Cells	0.60	0.59	0.66	0.46	

Appendix XXIX

Correlation Matrix between live weight and semen parameter of turkey toms fed 20% CP diet.

	Live Weight	Volume	Concentration	% Live	% Normal
Live Weight		0.61	0.78	0.65	0.50
Volume	0.61		0.77	0.72	0.62
Concentration	0.78	0.77		0.79	0.74
% Live Cells	0.65	0.72	0.79		0.48
% Normal Cells	0.50	0.62	0.74	0.48	

Appendix XXX

Mean values of blood parameters of turkey toms fed varying Levels of protein diets.

Groups	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
PCV (%)	29.20 ± 2.85 ^a	33.80 ± 1.39 ^b	32.60 ± 1.54 ^b
HB (g/dL)	7.90 ± 1.90 ^a	11.24 ± 0.47 ^b	10.84 ± 0.51 ^b
WBC (X10 ³ /μL)	3.32 ± 0.68 ^a	6.08 ± 2.37 ^b	3.80 ± 0.51 ^a
TOTAL PROTEIN	4.60 ± 0.44	4.24 ± 0.50	4.32 ± 0.27
HETEROPHILS (%)	9.40 ± 1.03	7.60 ± 2.25	8.80 ± 1.74
LYMPHOCYTES (%)	90.40 ± 1.17	91.60 ± 2.50	88.80 ± 0.97
MONOCYTES (%)	1	2	3
EOSINOPHILS (%)	0	1	3
BASOPHILS (%)	0	0	0
BAND CELLS (%)	0	0	0

Values with different superscripts (across the rows) differ significantly (P < 0.005).

Appendix XXXI

Mean Live weights of turkey toms fed varying levels of protein diets

	Live Weights (kg)		
	Group 1(12% CP)	Group 2(16% CP)	Group 3(20% CP)
Week 1	4.20	4.60	4.65
Week2	4.20	4.40	4.50
Week3	4.35	4.70	4.75
Week4	4.30	4.65	5.00
Week5	4.50	4.75	5.40
Week6	5.00	5.15	5.55
Week7	5.25	5.45	5.65
Week8	5.60	5.70	5.75
Week9	5.95	5.80	5.65
Week 10	6.30	5.90	6.15
week 11	6.40	6.05	6.55
Week 12	6.45	6.20	6.65
Week 13	6.25	6.65	6.90
Mean ± SEM	5.29 ± 0.25	5.39 ± 0.20	5.63 ± 0.22

P > 0.05