EFFECTS OF NEWCASTLE DISEASE VIRUS ON SOME REPRODUCTIVE PARAMETERS OF SHIKABROWN COCKS

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SEPTEMBER, 2007

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Department of Veterinary Surgery and Medicine
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SEPTEMBER, 2007

DECLARATION

I declare that the work in the dissertation entitled, "*Effects of Newcastle Disease Virus on some Reproductive Parameters of Shikabrown Cocks,*" has been performed by me in the Department of Veterinary Surgery and Medicine under the supervision of Professor P.A. Abdu, Professor L.O. Eduvie and Professor P.I. Rekwot. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation has previously been presented for another degree or diploma at any University.

_____ _____

Joseph Sankey RWUAAN
Candidate

Date

CERTIFICATION

This dissertation titled "*Effects of Newcastle Disease Virus on some Reproductive Parameters of Shikabrown Cocks*", by Joseph Sankey Rwuaan, meets the regulation governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University Zaria, and it is approved for its contributions to scientific knowledge and literary presentation.

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Prof. L.O. Eduvie Member, Supervisory Committee	Date
Prof. P.I. Rekwot Member, Supervisory Committee	Date
Prof. A. B. Ogunkoya Head, Department of Veterinary Surgery and Medicine	Date
Prof. S.A. Nkom Dean, Postgraduate School AHMADU BELLO UNIVERSITY, ZARIA	Date

DEDICATION

This dissertation is dedicated to the Almighty God, who sustains me and my family.

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ABSTRACT

Fifty red and white Shikabrown cocks were purchased and fed on a diet of layers mash with 18.0% crude protein, 95.6% dry matter, 17.1% crude fibre and 3.0% nitrogen. The experiment took place betwen January and May, 2005.

Twenty-five of the cocks were infected with $10^{6.0}EID_{50}$ of a velogenic Kudu strain Newcastle disease virus while twenty-five served as control. The cocks had their packed cell volume, total protein, hemagglutination inhibition ND antibody titres (HIT), semen characteristics, testosterone profiles and gonadal sperm reserves and body and organ weights determined weekly for six weeks post infection. At six weeks post-infection, twenty control and twenty infected cocks were slaughtered. Their internal organs were examined *in situ* and later removed and weighed; the dimensions of the testes were also taken. The semen was evaluated for volume and colour; and the latter was graded as: grade 1 = creamy; 2 = milky and 3 = watery.

There was no significant difference in the packed cell volume of the control and infected red and white cocks. The values of the total protein did not show any significant difference between the control and infected red and white cocks. The antibody titres of the control red and white Shikabrown cocks were significantly ($P<0.05$) lower than the antibody titres of the infected red and white cocks.

The cloacal temperatures and body weights did not show any significant difference between the red and white cocks. The semen volume of infected red cocks showed a general increase over that of the control red cocks. The semen volume of the control white cocks was significantly higher than that of the infected white cocks. The white

Shikabrown cocks had higher semen volume than the red Shikabrown cocks. The red cocks had slightly better semen colour than the white cocks. The control white cocks had better spermatozoa motility than the infected white cocks, while the infected red cocks had better spermatozoa motility than the control cocks.

The spermatozoa concentration of the control white cocks was consistently better than that of the infected white cocks. The spermatozoa concentration of the infected cocks was lower than that of the control cocks. The white cocks had better spermatozoa concentration than the red cocks.

The control white Shikabrown cocks had significantly ($P<0.05$) higher per cent live spermatozoa than the infected white cocks. The infected red cocks had significantly ($P<0.05$) lower per cent live spermatozoa than the control red cocks. The control white cocks had significantly ($P<0.05$) higher per cent live spermatozoa than the control red cocks.

The infected red and white Shikabrown cocks had higher percentage total spermatozoa abnormalities than the control red and white cocks. The mean testosterone concentration of the red and white cocks decreased post-infection from week 1 to 6.

The gonadal sperm reserves of the control red and white cocks were not significantly different but the gonadal sperm reserves of the control white cocks was significantly ($P<0.05$) higher than the gonadal sperm reserves of the infected red and white cocks. The total gonadal sperm reserves of the control white cocks was significantly ($P<0.05$) higher than the total gonadal sperm reserves of the infected red and white cocks. The left testicles of the control red and white cocks were significantly ($P<0.05$) heavier than those of the infected red and white cocks.

The liver, spleen, thymus, heart and adrenal glands of the infected red Shikabrown cocks were heavier than those of the control red Shikabrown, and white cocks and infected white Shikabrown. The weights of the spleen and brain were not signifiantly different in both the control and infected red and white cocks. The bursal weight of the control white cocks was heavier than that of control red, infected red and infected white cocks. The control red cocks had the heaviest adrenal glands. The infected red cocks had the highest liver body weight ratio. The spleen body weight ratio was the same for both control and infected red and white cocks. The control white cocks had the least thymus body weight ratio. The infected red cocks had the highest heart body weight ratio. The brain body weight ratio was the same for both control and infected cocks. The control white cocks had a higher bursa body weight ratio than the control red and infected red and white cocks. The infected red cocks had the highest adrenal body weight ratio. Only one infected white cock showed perivascular infiltration of lymphocytes and foci of glial cells.

It can be concluded that challenging the cocks with the velogenic Newcastle disease virus led to the cocks having higher antibody titres which gave them more protection. The infected red Shikabrown cocks had slightly higher antibody titres than the infected white Shikabrown cocks hence they were more protected from the velogenic Newcastle disease virus. The white cocks had better semen quality than the red cocks but more prone to the Newcastle disease virus. In a Newcastle disease endemic environment like Zaria farmers keeping Shikabrown chickens should adequately and routinely vaccinate them against the disease.

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LIST OF ABBREVIATIONS

ABU : Ahmadu Bello University
ANOVA : Analysis of variance
BWT : Body Weight
EDS : Egg drop syndrome
EEEV : Eastern Equine Encephalitis Virus
EID : Egg Infective Dose
FAO : Food and Agricultural Organisation
HA : Hemagglutination
HI : Hemagglutination inhibition
HIT : Hemagglutination inhibition tests
HJV : Highland J virus
IBR : Infectious Bovine Rhinotracheitis
IPV : Infectious Pustular Vulvovaginitis
LH : Luteinising Hormone
NAPRI : National Animal Production Research Institute
ND : Newcastle disease
NDV : Newcastle Disease Virus
NDV_4HR : Heat resistant Newcastle disease virus
Pc ABN : Percentage total spermatozoa abnormalities
Pc LIV : Per cent live spermatozoa
PCV : Packed cell volume
PMV : Paramyxovirus
RBCs : Red blood cells
RSB : Red Shikabrown
RSB_1 : Control Red Shikabrown
RSB_2 : Infected Red Shikabrown
SMEDI : Stillbirths Mummified Fetuses Embryonic Deaths and Infertility
TP : Total protein
TRT : Treatment
UV : Utero vaginal
WBCs : White blood cells
WSB : White Shikabrown
WSB_1 : Control White Shikabrown
WSB_2 : Infected White Shikabrown

CHAPTER 1

INTRODUCTION

1.1 GENERAL BACKGROUND

The population of chickens in Africa has been estimated to be 460 million (Jahnke *et al*., 1987). The FAO estimated that the animal protein intake from Nigeria livestock is less than 50% of the recommended target of 35.0 gms/head/year of animal based proteins (David-West, 1985). There are about 134 million chickens in Nigeria of which 124 million are indigenous local chickens (Akinwumi *et al*., 1979; Nawathe and Lamorde, 1982). Resources Inventory and Management (RIM) report of 1993 indicated that the indigenous local breed consisted of 102.8 million out of 150 million poultry in Nigeria. Usman (2002), reported that 84.5% of local chickens are found in the Northern part of Nigeria. Similarly 83% of the exotic chickens are found in the Southern part of Nigeria (Majiyagbe and Lamorde, 1997). These local chickens are found in small numbers of between 5 and 20 or more per household. They wander around scavenging for food with minimal or no prophylactic treatment or vaccination against major diseases like Newcastle disease, Gumboro disease, etc. (Adu *et al*., 1986; Copland, 1987; Musiime, 1992; Olabode *et al*., 1992; Dipeolu *et al*., 1998; Orajaka *et al*., 1999). These chickens have been reported to act as potential reservoirs and carriers for all breeds, especially the exotic breeds, which are very susceptible to most of the poultry diseases found locally. Most of the exotic breeds in Nigeria are in commercial farms (Adu *et al*., 1986; Alders and Spradbrow, 2001). Rural chickens are kept by majority of households and contribute to the national economy and help in alleviating poverty (Nawathe *et al*., 1975; Jagne *et al*., 1991; and Spradbrow, 1993).

Reproductive diseases impair reproductive activities and thus, reduce productivity in livestock, apart from the general debilitating effects on the health of an animal (Zemjanis, 1974). Among the reproductive diseases reported for livestock in Nigeria are brucellosis (Nuru, 1974; Bale and Kumi-Diaka, 1981), scrotal onchocerciasis (Akusu *et al*., 1983), trypanosomosis (Ogwu, 1983), campylobacteriosis (Bawa *et al*., 1987 and 1991) and dermatophilosis (Kumi-Diaka *et al*., 1980; Sekoni, 1983 and 1993). These diseases are known to cause infertility in the form of irregular cycles, embryonic mortality and abortions. Dermatophilosis has been reported to cause sterility in bulls through its effects on testicular thermoregulation which results from scrotal insulation caused by the scabs formed by the infection (Kumi-Diaka *et al*., 1980; Sekoni, 1983 and 1993). Semen quality deteriorated progressively with drastic decreases in sperm concentration, volume of semen and increased abnormal spermatozoa of bulls infected with trypanosomes (Sekoni *et al*., 1988b).

A number of diseases affect reproduction in poultry. Egg drop syndrome (EDS) is a viral disease of laying hens characterized by production of thin-shelled or shell-less discoloured eggs in apparently healthy birds (Nawathe and Abegunde, 1980). Pullorum disease caused by Salmonella pullorum causes sub-optimal egg production, fertility and hatchability in chickens and turkeys. Infectious bronchitis virus affects the production and quality of eggs while infectious avian encephalomyelitis virus causes reduction in egg production temporarily in laying hens; it also causes a lowered hatchability of fertile eggs (Jordan, 1990). Egg transmission of the virus occurs from infected to susceptible stock housed together. In infectious laryngotrocheitis infection, egg production drops and ceases completely for sometime without a loss of egg quality.

Avian mycoplasmosis caused by *Mycoplasma gallisepticum* causes sub-optimal egg production in layers accompanied by reduced hatchability of chicks and poults and also causes salpingitis in chickens (Jordan, 1990). *Mycoplasma meleagridis* and *Mycoplasma iowae* infections lead to reduced hatchability in turkey hens (Jordan, 1990).

A lot of work has been done on the male chicken in the areas of reproductive physiology and artificial insemination (Etches, 1996). While NDV has been reported to cause obvious reproductive disorders in the hen (Abdu *et al*., 2006), the effects of the virus on the male appear not to have been documented. Therefore this research was conducted to determine the effects of a velogenic NDV on:

(1) Packed cell volume (PCV), total protein and hemagglutination inhibition ND antibody titres (HIT).

(2) Semen characteristics of Shikabrown cocks.

(3) Testosterone profiles and gonadal sperm reserves of these cocks.

(4) Body and organ weights.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Newcastle disease is a highly contagious generalized fatal viral disease of domestic poultry and wild birds characterized by gastro-intestinal, respiratory and nervous signs (Geering *et al*., 1995; Morgan *et al*., 1998). It is considered the most infectious disease of birds and poultry. Many birds die before showing any clinical signs because of its virulence. A hundred per cent mortality can occur in a farm (Texas Animal Health Commission, 2004).

Nine serogroups of avian paramyxovirus have been recognized – PMV-1 to PMV-9 (Alexander, 1980; 1986; 1988). Of these Newcastle virus serogroups, PMV-1 remains the most important pathogen for poultry. Newcastle disease may vary widely in the type and severity in the disease it produces. The effect of Newcastle disease on reproduction in the male chicken has not been documented. Newcastle affects egg production in the female poultry (Abdu *et al*., 2006). In the hen, it causes a drop in egg production. Other viruses affect production of eggs in the female, such as infectious bronchitis, which affects the production and quality of the eggs (Jordan, 1990). Infectious avian encephalomyelitis virus causes reduction in egg production temporarily in laying hens; it also causes a lowered hatchability of fertile eggs (Jordan, 1990). Egg transmission of the virus occurs from infected to susceptible stock housed together. Pullorum disease caused by *Salmonella pullorum* causes suboptimal egg production. In infectious laryngotracheitis (ILT) egg production drops and ceases completely for sometimes. In uncomplicated cases egg production returns to normal without loss of egg quality (Jordan, 1990). Avian mycoplasmosis caused by *Mycoplasma*

gallisepticum infection causes suboptimal egg production in layers accompanied by reduced hatchability of chicks and poults and causes salpingitis in chickens (Jordan, 1990). *Mycoplasma meleagridis* (Mm) infection causes reduced hatchability in turkeys. *Mycoplasma iowae* (Mi) infection in turkeys' causes reduced hatchability in turkey hens. The organism is transmitted through the egg which becomes infected during passage through the oviduct. The disease can be spread by artificial insemination using infected semen from the turkey tom (Jordan, 1990). A variety of viruses cause reproductive problems in domestic animals. The virus causing Infectious Bovine Rhinotracheitis (IBR) causes infectious pustular vulvo-vaginitis (IPV) in cattle. Lesions occur in both female and male genital tracts where vesicles and pustules occur on the mucosa of the vulva, prepuce and penis. In the bull, there may be small amount of exudate and some adhesions. The bull usually recovers in about two weeks. Some infected bulls have orchitis and this affects their reproductive efficiency (Roberts, 1971).

In Equines, Equine Herpes Virus rhinopneumonitis virus causes abortion in mares and ulcers on the glans penis of the stallion. Equine arteritis or epizootic cellulites – pinkage virus and equine infectious anaemia virus also cause abortion in mares.

In sheep and goats rift valley fever virus, foot and mouth disease, blue tongue, rinderpest and enzootic abortion of ewes or ovine virus cause abortion in ewes and goats.

In swine, Hog cholera, pseudorabies, various picorna or Stillbirths, Mummified Fetuses Emryonic Deaths and Infertility (SMEDI) viruses cause abortion. The use of artificial insemination using infected semen helps in spreading some of these viral diseases, hence semen used for artificial insemination should be well checked before use (Roberts, 1971).

In many countries there are many strains of the ND virus that affect poultry and wild birds. This ranges from non-pathogenic to highly virulent strains (Alexander, 1980). Rural people that engage in poultry business suffer from huge loses due to Newcastle disease and as a result get discouraged from paying proper attention to their flocks. This happens more commonly during the cold harmattan period of the year, November – February (Nawathe, 1988; Okeke and Lamorde, 1988; Wosu, 1992; Ibrahim and Abdu, 1992). Okeke and Lamorde (1988) reported that about 200 – 250 outbreaks of ND are reported annually in Nigeria with an average mortality rate of 33.0% and 56.5% in adults and chicks exposed respectively. The clinical signs include anorexia, sneezing, coughing, rales, torticollis, leg paralysis, greenish diarrhea and death (Nawathe, 1988). Death can occur suddenly without any preceeding signs and layers suddenly stop egg production but may resume gradually after 3–4 weeks period (Okeke and Lamorde, 1988). Newcastle disease is also found in domestic chickens, turkeys, pheasants, pigeons, quails and guinea fowls. Ducks and geese are susceptible but severe disease is rare (Geering *et al*., 1995). This disease is so important that it is known by different names in different communities. In Nigeria ND is known as "*Haukan Kaji*" (Hausa) in northern Nigeria (Ibrahim and Abdu, 1992); "*Koli"* (Yoruba) in Western Nigeria (Dipeolu *et al*., 1998) and in Igbo it is called "*Ogbunankpo*" (Nwanta, 2003). In western region of Ghana, ND is known as "*Konoku, Twase Obgo*" in the greater Accra region; "*Adza*" in Volta region and "*Nkoko Yare"* in Twi language, in Mozambique, ND is known as "*Nuzugo*" in the Shangen language and "*Mbendi*" in shona (Alders and Spradbrow, 2001). In Asia ND is known as "*Ranikhet*" disease (Alders and Spradbrow, 2001). Many species of wild birds are susceptible. Psittacines

(Parrots) are highly susceptible and can excrete the virus for long periods (Alexander, 1998). Breed or genetic stock appears to have very little effect on the susceptibility of chickens to the disease. Natural routes of infection are nasal, oral and ocular and they appear to emphasize the respiratory nature of the disease, while intramuscular, intravenous and intracerebral routes appear to enhance the neurologic signs (Alexander, 1988). Newcastle disease virus is relatively heat stable and survives well in protected environments. Coughing is not required to produce infected aerosols (Alexander, 1988).

2.2 HISTORY AND REVIEW OF NEWCASTLE DISEASE

The first reported outbreak of what is considered to be Newcastle disease took place in Java Indonesia in 1926 (Kraneveld, 1926) and in Newcastle upon-tyne in 1927 (Doyle, 1927). Disease outbreaks similar to what is now recognised as ND took place in Central Europe; these predated 1926 (Halasz, 1912). Levine (1964), indicated that the disease may have been present in Korea as early as 1924. Doyle in 1935 coined the name Newcastle Disease (ND) to distinguish it from other similar diseases. It later became clear that there were other diseases less severe than ND caused by viruses indistinguishable from Newcastle disease virus. The taxonomy and nomenclature of the family Paramyxoviridae has been modified and now has four genera forming two sub-families (Rima *et al*., 1995). The sub-family paramyxoviridae has three genera. Rubulavirus which includes NDV and the other avian paramyxoviruses; paramyxovirus and morbillivirus. Newcastle disease virus may vary widely in the type and severity of the disease it produces (Alexander, 1980). Newcastle disease has been termed pseudo fowl, pest, avian distemper, pseudovogel–pest, atypische, Geflugelpest, pseudo poultry plague, avian pest, Ranikhet disease, Tetelo disease, Korean fowl plague and avian pneumoencephalitis (Alexander, 1980). The other avian paramyxoviruses have

very few synonyms (Alexander, 1980). Newcastle disease affects both domestic and wild birds and has become a major problem in many countries where poultry are reared (Allan *et al*., 1978; Ezeibe and Ozoemena, 2000; Alders and Spradbrow, 2001). The viral particles are between 100 and 300 millimicrons in diameter (Waterson and Cruickshank, 1963). The envelope is found to contain myxovirus fringe consisting of hemagglutinin and neuraminidase protein which binds to receptors on the surface of avian red blood cells (Alexander, 1997). This properly and specific inhibitor of agglutination by antisera are proven powerful tools in the diagnosis of the disease (Burnet, 1942).

Chicken red blood cells (RBCs) are usually used in hemagglutination (HA) test but NDV will cause agglutination of amphibian, reptilian, avian, human, mouse and guinea pig RBCs but the ability to agglutinate cattle, sheep, goat, swine and horse cells varied with the strain of NDV (Winslow *et al*., 1950; Lancaster, 1975; and Alexander, 1997). Below the projections on the viral lipid envelope is a layer of lipid and the use of lipid solvent causes the desegregation of this layer and the disruption of the viron (Allan *et al*., 1978).

Newcastle disease virus is a single stranded RNA virus (Alexander, 1997). The virus is readily inactivated by formalin, alcohol, methiolate, lipid solvents, lysol, heat and pH effects (Lancaster, 1975; Allan *et al*., 1978; Beard and Hanson, 1984). The rate at which infectivity is destroyed is found to be dependent on the strain of the virus, the length of time of exposure, the quantity of the virus and the nature of the suspending medium and interactions between treatments (Alexander, 1997). No single treatment has been able to destroy all the virus but such treatment may result in a low probability of infective virus remaining.

2.3 EPIDEMIOLOGY OF NEWCASTLE DISEASE IN POULTRY

In non-immune intensively managed commercial poultry, the introduction of a pathogenic strain of NDV is enough to infect the birds and is capable of causing ND (Martin, 1992). In rural poultry the occurrence of ND is dependent on a combination of factors. These include the presence of pathogenic strain of NDV which is necessary for the disease to develop but is not in itself a sufficient cause of the disease (Martin, 1992). This is due to the fact that rural local poultry populations are mixed in terms of susceptibility to infection with NDV because of immunity due to age and exposure to NDV. In extensive conditions, the spread of NDV from bird to bird does not occur as readily as in intensive conditions where a large number of poultry are housed together. Other factors that affect the ease of spread of NDV and the susceptibility of birds are important in the epidemiology of ND in rural village poultry (Martin, 1992).

In many countries with high population of rural poultry, ND is an important infectious disease which may be endemic within individual regions or villages (Bell and Mouloudi, 1988). Control is possible but efficient application of vaccines and rigorous biosecurity are required (Alders and Spradbrow, 2001). The epidemiology of ND is fairly well understood in commercial poultry. The factors that influence the spread between flocks can be checked with physical and chemical barriers (Martin, 1992). Vaccination stops the spread within a flock.

Clinical signs of ND are very variable depending on the strain of the virus, species and age of bird, concurrent disease and pre-existing immunity. Four broad clinical syndromes are recognized (Geering *et al*., 1995; Morgan-Capner and Bryden, 1998).

These include viscerotropic velogenic, neurotropic velogenic, mesogenic and lentogenic (Geering *et al*., 1995; Morgan-Capner and Bryden, 1998).

(1) *Viscerotropic Velogenic:* In this form the signs recognized are: sudden appearance, rapid spread, marked depression, loss of appetite, sharp drop in egg production, increased respiration, profuse bright green diarrhea, edematous swellings of the head and cyanosis of the combs and conjuctivitis, prostration with many birds dying within a few days. Nervous signs are shown in those that survive initial phase and there is high mortality (>90%) in susceptible flocks.

(2) *Neurotropic Velogenic*: In this form, acute respiratory and nervous signs predominate, there is sudden depression, loss of appetite, drop in egg production, respiratory distress with severe coughing and also gasping, nervous signs such as head tremors, wing and leg paralysis, torticollis, mortality rate in adults is 10 – 20%. Mortality may be much higher in young chickens.

(3) *Mesogenic:* Depression, weight loss, drop in egg production and quality (lasting 1–2 weeks) are observed in this form. Acute respiratory disease with coughing, gasping, nervous signs may develop late in the clinical course. Mortality rate is about 10%.

(4) *Lentogenic:* Commonly subclinical, mild respiratory signs, temporary loss of appetite, drop in egg production, no nervous sign. Negligible mortality unless concurrent disease is present in this form.

2.4 RESERVOIR HOSTS

Turkeys, ducks, doves, guinea fowls, and wild birds may act as sources of infection for the domestic chicken (Lancaster, 1975). The greatest potential for the spread of NDV is by humans and their equipment. Humans may be infected in the conjunctival sac with the NDV virus which could pose a method of spread, but more important is the mechanical transfer of infective material, e.g. feces. Vaccination crews moving from farm to farm have been implicated in the spread of the disease (Utterback and Schwartz, 1973). Chicken carriers and partially immune chicken can develop infection with velogenic NDV and display mild or no clinical signs; they can shed the virus for up to 5 weeks. Wild birds harbour velogenic, mesogenic and lentogenic strains of NDV and hence shed the virus (Lancaster, 1975; Hanson, 1976 and 1978; Alexander, 1988; Okeke and Lamorde, 1988). Physical environment plays a role as a reservoir of NDV in villages (Lancaster, 1975). The virus can survive for 3 months at temperature of 20 - 30°C and longer at cooler temperature when shed by infected birds. Infection can be spread from dried feces or dusty materials containing NDV (Lancaster, 1975).

The incubation period of Newcastle disease after natural exposure has been reported to vary from 2 to 15 days (average 5 – 6 days). The speed with which signs appear is variable depending on the infecting virus, the host species, its age and immune status, infection with other organisms, environmental conditions, the route of exposure and the dose (Alexander, 1988).

2.5 METHODS OF TRANSMITTING THE NEWCASTLE DISEASE VIRUS

Chickens are susceptible to infection by both inhalation and ingestion of NDV (Spalatin and Hanson, 1976; Beard *et al*., 1970; Utterback and Schwarz, 1973; Lancaster, 1975; Lancaster and Alexander, 1975; Nawathe *et al*., 1975; Alexander *et al*., 1984; Alexander, 1988; Alders and Spradbrow, 2001). Chickens are more susceptible to NDV reaching the respiratory tract, but this may not be the case for all strains of ND. Strains vary in tropism for organs and some strains seem adapted to fecal–oral spread (Alders and Spradbrow, 2001). Spread in a flock is probably by aerosol within the crowded community, but airborne virus may travel long distances. Alexander (1988) concluded that infection may take place by either inhalation or ingestion and that spread from one bird to another depends on the availability of the virus in an infectious form. Infectious virus may be present in aerosols;, birds placed in an atmosphere containing such aerosols become infected. Estimates of airborne spread have been reported to extend up to 40 kilometres (Alders and Spradbrow, 2001). Contaminated human shoes and clothing, crates, eggs and contaminated food and food containers, drinking water, vaccines, other animals and poultry products help in spreading the disease (Majiyagbe and Nawathe, 1981; Nawathe, 1988; Alders and Spradbrow, 2001). Rural chickens roam about the vicinity of their houses, they move together in small groups and are either in direct or indirect contact. In the evening, they move together to where they will spend the night either in houses or on trees. Chickens that are housed at night are more susceptible to ND (Huchzermeyer, 1993). Infection occurs by close contact where sick birds will infect

healthy ones. Birds that perch on trees to roost for the night have less chances of getting infected because the sick birds cannot reach them. Brooding hens and hens with clutches of chicks also escape infection because they are segregated (Huchzermeyer, 1993). Huchzermeyer (1993), explained that infection occurred more rapidly at night in the absence of solar radiation. Intense sources of aerosols and virus are not available in rural areas but these are possible in commercial farms found mostly in cities. Movement of infected chicken is probably the main source of virus (Nawathe, 1988; Ibrahim and Abdu, 1992; Alders and Spradbrow, 2001). Rural chickens pass through markets, they congregate and spread NDV and disperse. People in the rural areas are aware that outbreaks of ND may follow introduction of a newly purchased or loaned chicken (Ibrahim and Abdu, 1992). Many people in the villages are also aware of the devastating infectious nature of Newcastle disease and the lack of treatment for the disease (Ibrahim and Abdu, 1992; Awan *et al*., 1994; Mavale, 2000). Dead birds are thrown away exposed instead of being buried and sick birds are slaughtered for consumption or sold. The sale of infected birds aids in the spread of infection (Nawathe, 1988; Alders and Spradbrow, 2001). Seasonal conditions have been attributed to the outbreaks of ND (Nawathe, 1988; Ibrahim and Abdu, 1992). The frequency of outbreaks is directly related to the size of the poultry population in a particular area. In Indonesia household chickens are sold to purchase rice seeds for planting (Alders and Spradbrow, 2001). This movement of birds can influence the spread of ND. Outbreak of ND occurs in Uganda during the dry season to coincide with the travel of unemployed agricultural workers who carry chickens as gifts when they visit relatives (Alders and Spradbrow, 2001). Outbreaks also occur

during festivities, e.g. Christmas, New Year and Sallah when large numbers of chickens are sold (Ibrahim and Abdu, 1992; Alders and Spradbrow, 2001). In the dry season food is scarce and chickens are readily sold to buy food and other needed items. These chickens may be infected with NDV and thus serve as vehicles for spreading the ND to susceptible flocks. It has been observed that in flocks where ND outbreaks occur the chickens are sold to offset loses hence encouraging the spread of the disease (Alexander, 1988; Cherdechai, 1988). Virus strains which vary in pathogenecity, from velogenic to apathogenic aid the spread of Newcastle disease. In Nigeria, velogenic strains have been commonly isolated in various poultry species (Majiyagbe and Nawathe, 1981; Onunkwo and Momoh, 1981; Echeonwu *et al*., 1993). The characteristics of NDV as enumerated above makes it difficult for one to generalize in respect of the conditions favouring outbreaks of ND among poultry especially in rural environments where the situation may be complicated by the presence of several other strains of NDV (Martin, 1992).

Newcastle disease virus may persist in undispersed chicken faeces for more than six months (Alexander *et al*., 1985). Under village conditions the virus is unlikely to survive outside a host for longer than one month (Alexander, 1997). Local chickens act as reservoirs of Newcastle disease virus in different ways (Bell and Mouloudi, 1988). Birds that recover from the disease may shed the virus for some time after recovery (Awan *et al*., 1994). Birds with latent infection may also act as reservoirs of the disease. This happens when chickens are infected by mesogenic or avirulent strains of the virus or are partially immune (Mavale, 2000). In birds that have been vaccinated mild or hidden infection with virulent strains of the virus may develop and

these birds may then become a source of infection to fully susceptible chickens. This has been reported to be one of the most important sources of infection (Bell and Mouloudi, 1988; Awan *et al*., 1994; Mavale, 2000). Nawathe (1988), Okeke and Lamorde (1988). Awan *et al*. (1994) and Mavale (2000), have documented the role of ducks, domestic mammals, wild birds and wild mammals in the spread and maintenance of NDV within an environment. Some of these animals maintain close contact with domestic birds thus providing an opportunity to spread the disease. In viewing the modes of transmission between birds, Alexander (1988) concluded that infection may take place either by inhalation or ingestion and that spread from one bird to another depends on the form. It is tempting to assume that NDV is primarily transmitted by fine aerosols or large droplets that are inhaled by susceptible birds. Evidence to prove this is however lacking. Infectious virus may be present in aerosols and birds placed in an atmosphere containing such aerosols become infected. This is the basis for mass application of live vaccines by spray and aerosol generators (Meulemans *et al*., 1988; Steven *et al*., 1976). In naturally occurring infections, large and small droplets containing virus will be released from infected birds as a result of replication in the respiratory tract or as a result of dust and other particles including faeces (Alexander, 1988). These virus laden particles may be inhaled.

During the course of infection of birds by NDV large amounts of the virus are excreted in the faeces. Ingestion of the faeces results in infection; this is how the disease likely spreads for avirulent enteric NDV and the pigeon variant virus from bird to bird (Alexander *et al*., 1984). Neither of these diseases produces respiratory signs in infected birds. Passage of infection from parent to progeny via the embryo (vertical transmission) is controversial. Experimental assessment using virulent viruses is usually hampered by

cessation of egg lying in infected birds (Alexander, 1988). Infected embryos have been reported during natural infections of laying hens with virulent virus (Lancaster and Alexander, 1975; Beard and Hanson, 1984). This generally results in the death of the infected embryo during incubation. Cracked or broken infected eggs may serve as a source of NDV for newly hatched chicks, the same applies to virus laden faeces that contaminate the outside of the egg. Virus may also penetrate the outside of the egg after laying (Williams and Dillard, 1968). This complicates the assessment of true vertical or transovarian transmission. Infected chicks may be hatched from eggs infected with vaccinal or other lentogenic viruses that do not necessarily cause the death of the embryo (Coman, 1963; French *et al*., 1967). In naturally occurring infections it is not clear how the embryos become infected. Lasota vaccine has been shown to be present in most of the reproductive organs after vaccination (Raszewska, 1964). In Nigeria the probability of aerosol transmission has been reported to be minimal (Usman, 2002). However, where shelter is provided for roosting, this aerosol transmission becomes important because birds in a limited space can release infectious aerosol droplets that may be inhaled by other birds in the shelter. This route of transmission is important in intensively managed birds (Mavale, 2000).

The spread of NDV both within and between local flocks is slower than that found in intensive or semi-intensive systems. The disease can take weeks to pass through the flock and months to pass through the village (Awan *et al*., 1994). Lublin *et al*. (2001) in Israel reported a velogenic strain of ND in a bearded female vulture (*Glypaetus barbatus*) that was found dead in the research zoo of Tel Aviv University, Israel. Murakawa *et al*. (2000), isolated a NDV named MET 95, this strain was determined to be a lentogenic NDV. The strain had the property of eluting rapidly at 4°C and has low

thermostability in hemagglutinating activity with chicken erythrocytes. They found the MET 95 strain a promising candidate as a live ND vaccine strain. In Taiwan Jui-Pinchen and Ching-Ho Wang (2002) studied the sporadic spread of ND disease during the period 1998-2000. They found that in some cases the disease occurred in broilers less than two weeks old that originated in a broiler breeder farm, hence spread of the disease from the infected breeder farm to broiler farms was suspected. From their study they confirmed that a chicken embryo infected in vivo with a low titre of NDV can hatch and contain NDV after hatching which results in NDV spreading through eggs. Pospisil *et al*. (1991), demonstrated the presence of lentogenic virus in chick embryos and young progeny including day old chicks. Capua *et al*. (1993), isolated virulent NDV from cloacal swabs taken from birds that had laid eggs in which NDV was isolated from the embryos.

The most common route of transmission in rural chickens is probably the oral route, through the ingestion of contaminated feeds, water and feces from infected animals within the immediate vicinity (Mavale, 2000). Other factors that play a role in the slow spread of NDV in rural chickens include flock density and consequent low contact rate as well as the low immune status of local rural poultry (Awan *et al*., 1994; Mavale, 2000).

2.6 EPIDEMIC AND ENDEMIC NEWCASTLE DISEASE

In the epidemic outbreak, susceptible flocks in the whole area become infected within a short time and mortalities are high and sometimes total (Alders and Spradbrow, 2001). Chickens that survive become resistant during subsequent outbreaks.

In endemic areas the NDV is prevalent in partially immune population and slowly spreads among the susceptible population of the flock causing occasional deaths which are not stressful to the flock owners.

Alders and Spradbrow (2001) stressed the impact of vaccination on the epidemiology of Newcastle disease. Vaccine application alters the epidmiology of the disease such that it will prevent disease but not infection. Vaccinated birds exposed to a virulent NDV will not develop clinical signs. Replication of the infecting virus will occur and birds will excrete virulent NDV (Alders and Spradbrow, 2001).

2.7 INCIDENCE OF NEWCASTLE DISEASE IN NIGERIA

Newcastle disease was tentatively diagnosed in Eastern Nigeria in 1951 and laboratory confirmation of the disease took place in Vom in 1953 (Hill *et al*., 1953; David West, 1972). Since then ND has been devastating the country's poultry industry (Fatumbi and Adene, 1979; Ugochukwu, 1982). Huge economic loses have been incurred by poultry farmers due to deaths of chickens and fall in egg production in endemic areas (David-West, 1979). Field outbreaks of ND are grossly under reported especially in the rural areas where about 90% of Nigeria's poultry population abound (Ugochukwu, 1982; Patrick *et al*., 1987).

Velogenic strains are commonly isolated in Nigeria (Nawathe *et al*., 1975; Majiyagbe and Nawathe, 1981; Patrick *et al*., 1987; Echeonwu *et al*., 1993). Velogenic viscerotropic strain has been isolated in Nigeria from a Parrot (Onunkwo and Momoh, 1981), which lends credence to the suggestion that wild birds could be sources of infection for chickens. Outbreaks of ND have been reported from various parts of

Nigeria (Kaschula, 1961; David-West, 1972; Ojo *et al*., 1973). Alexander *et al*. (1984). Reports by Nawathe *et al*. (1975), Majiyagbe and Nawathe (1981) and Echeonwu *et al*. (1993), confirmed that velogenic viscerotropic NDV strains are common in the rural areas of Nigeria causing high mortalities in unprotected birds.

Sero-epidemiological studies with respect to ND prevalence in both rural and commercial chickens have been documented in Nigeria. Adu *et al*. (1986), conducted studies on immunological status of Nigerian local chicken to ND and found 307 (41.0%) out of 748 local unvaccinated chickens were positive on screening for hemagglutination inhibition (HI) antibodies. This showed that these local chickens were immuno-competent following exposure to NDV. Adu *et al*. (1986) also isolated a virulent NDV from both HI positive and negative local chicken. Ezeokoli *et al*. (1984), in their study to determine the antibody prevalence rate for ND in different breeds of chickens under various management systems in Nigeria showed an overall prevalence rate of 77.0% with the large commercial farms having the highest prevalence rate of 80.0%, the free-range birds with a prevalence rate of 71.9%, backyard chicken had a prevalence rate of 62.9%. The same study, showed that age specific prevalence rate indicated that backyard management system involving birds of 16 – 24 weeks of age were at the highest risk of infection and that there was no clear breed specific tendencies. In Zaria, Halle *et al*. (1999), recorded 31.2% cases of ND outbreaks. Oranusi and Onyekaba (1986), in their serological survey of commercial flocks in the Niger Delta region of Nigeria stated that 163 (54.3%) out of 300 adult birds sampled had HI antibodies to NDV, and that out of 100 birds with history of complete ND conventional vaccination, 99% had titres of 1:5 or more, while 42.0% of the 100 birds that had only one vaccination with B1 vaccine had titres below 1:5 and 22.0% of the

100 birds with no history of Newcastle disease vaccination were positive reactors. Orajaka *et al*. (1999), revealed a high sero-prevalence of 63.0% and a high prevalence rate of NDV intensity in the dry harmattan season than in the wet season in the savannah zone of Southern Nigeria. Oyewole *et al*. (1996), in Ibadan reported a sero-prevalence rate of 38.0% and below in local chickens.

Fatumbi and Adene (1979), reported a morbidity rate of 80.0% in outbreaks of ND and an average mortality rate of 28.0% in local chickens. Abdu *et al*. (1992), reported that the disease is responsible for 56.0% of all mortalities in local chickens. Mortality due to ND ranges from 24% to 100% and that in most outbreaks 45% to 100% of the birds in a flock may die of the disease (Abdu *et al*., 1992).

2.8 DIAGNOSIS OF NEWCASTLE DISEASE

The flock history of respiratory signs, nervous disorders and other typical lesions are often sufficient to allow for a tentative diagnosis (Alexander, 1997). None of the clinical signs or lesions of ND can be regarded as pathogenomonic (Alexander, 1988). ND cannot be differentiated from other respiratory diseases such as infectious bronchitis, avian influenza, laryngotracheitis, mycoplasmosis and other encephalitic diseases like avian encephalomyelitis, botulism and poisoning such as DDT, except by laboratory tests such as serology and virus isolation (Hall *et al*., 1971; Okeke and Lamorde, 1988).

Antibody from the serum of convalescent birds and NDV culture can be identified using serological techniques such as virus or serum neutralization tests, enzyme-linked immunosorbent assay (ELISA), hemagglutination inhibition and hemagglutination tests (National Academy of Science, 1971; Rockborn *et al*., 1990; International Office

for Epizootics, 1996; Thayer and Beard, 1998). The hemagglutination inhibition test is the most widely used test for ND diagnosis at present and titres may be regarded as positive if there is inhibition at initial serum dilution of 1/16 (2^4 or 4 $1og_2$) or more against 4HA unit of antigen (OIE, 1996).

2.9 POSTMORTEM LESIONS

The gross lesions and the organs affected in birds infected with NDV are dependent on the strain and pathotype of the infecting virus in addition to the host species, age, immune status, coinfection with other organisms, environmental stress, social stress, route of exposure and the virus dose (McFerran and McCracken, 1988; Alexander, 1997). There are no pathognomonic lesions associated with any form of the disease and in some cases the absence of gross lesions may be reported (Alexander, 1997). Presence of hemorrhagic lesions in the intestine of infected chicken are used as distinguishing factors between velogenic viscerotropic ND and velogenic neurotropic NDV (Hanson *et al*., 1973; Hanson, 1980). These lesions are often particularly prominent in the proventriculus, ceca and small intestine. The lesions are markedly hemorrhagic and may appear to result from necrosis of the intestinal wall or lymphoid foci such as cecal tonsils. Generally it has been shown that gross lesions are not observed in the central nervous system of birds infected with NDV regardless of the pathotype (McFerran and McCracken, 1988). Gross pathologic lesions are not always in the respiratory tract, but when they are observed consist predominantly of hemorrhages and marked congestion of the trachea (Alexander, 1997). Air sacculitis are present at times even after infection with relatively mild strains and thickening of the air sacs with catarrhal or casious exudates often observed (Beard and Hanson, 1984). Chickens and turkeys

infected with velogenic NDV while laying usually reveal egg yolk in the abdominal cavity. The ovarian follicles are often flaccid and degenerative. Hemorrhage and discolouration of the reproductive organs may occur (Alexander, 1997).

2.10 HISTOPATHOLOGIC LESIONS

The histopathologic lesions seen in infected chicken varies and are dependent on the factors that affect the severity of the disease. Histologic changes are reported to concentrate in nervous, vascular, lymphoid systems, intestinal and respiratory tracts, reproductive system and other organs (Alexander, 1997).

2.11 IMMUNITY TO NEWCASTLE DISEASE INFECTION

Immunity develops following NDV infection or vaccination. Cell mediated immunity is the initial immune response to infection with NDV and has been shown to be detectable at about 2 – 3 days after infection with live vaccine strains (Timm and Alexander, 1977). This presumably explains the early protection against challenge recorded in vaccinated birds before a measurable antibody response is seen (Allan and Gough, 1976; Alexander, 1997).

Antibodies capable of protecting the host develop following vaccination or natural exposure to NDV. Alexander (1997), reported that when chickens survive NDV infection for a long period, antibodies are usually detectable in the serum within 6 – 10 days. Okeke and Lamorde (1988), reported that antibodies to ND appear within a period of 4 – 8 days following vaccination and immune levels largely depend on the infecting strain. Generally peak response appears at about 3 – 4 weeks. Alexander (1997), explained that decline in antibody titre varies with the titre achieved but much slower than their development. Hemagglutination inhibition (HI) antibodies have been reported to likely

remain detectable for up to one year in birds that recovered from infection with mesogenic viruses or after a series of immunization (Alexander, 1997). Reinfection or immunization some weeks after the titre begins to decline produced a secondary response (Allan *et al*., 1978b).

Ambali *et al*. (1998), concluded an investigation into the serological response of guinea fowl to two lentogenic vaccines, NDV Lasota and ND heat resistant (NDV_4HR) using HI test (HIT). The outcome revealed that birds vaccinated with NDV-Lasota responded poorly with the highest antibody titre of 4.2 log_2 obtained one week post vaccination after which the titre dropped to 2 log_2 by the fourth week. The response to NDV_4HR was found to be better as the titre rose gradually and by the fourth week post vaccination the highest titre of 9.5 log_2 was reached at the termination of the experiment. Usman (2002), in his vaccine trial on chickens using NDV_4HR and NDV-Lasota in drinking water, grains and their brans showed that both NDV-Lasota vaccinated and in contact chicks developed antibodies to ND with geometric mean titres ranging from 0.0 log_2 to 5.3 log_2 and 0.2 log_2 to 5.2 log_2 for vaccinated and in contact chicks respectively. The heat resistant NDV_4HR vaccinated chicks and those in contact had geometric mean titres ranging from 0.0 log_2 to 5.0 log_2 and 0.0 log_2 to 5.2 log_2 for vaccinated and in contact chicks respectively. Mortality records obtained on challenge of the chicks in the groups with virulent NDV Hert's 33/56 strain revealed 80% mortality rate in the heat resistant NDV_4 HR vaccinated chicks in ground millet and lowest mortality rate was recorded in chicks vaccinated using water. Mortality rate of 100% was observed in contact chicks with heat resistant NDV vaccine placed on guinea corn bran and maize bran (Usman, 2002).

Similarly, for the NDV-Lasota group, 60% protection rate was recorded in chicks vaccinated through guinea corn bran and 80% mortality rate recorded in contact chicks vaccinated through millet bran and 100% protection rate was observed in chicks vaccinated with NDV-La Sota and NDV_4HR in water. Rehimani *et al*. (1995) and Ideris *et al*. (1990), reported a strong correlation between level of passively acquired antibody following vaccination with NDV_4HR and survival of birds after challenge. Rehimani *et al*. (1995) also found that no chicken with a titre of antibodies greater than 5.3 log_2 succumbed to challenge with virulent NDV and that mortalities in other chickens varied from 88.0% to 25.0% for those with geometric mean titre of 4.3 log_2. The 88.0% mortalities had no detectable antibodies. Abdu *et al*. (1999), in their research to test the potency of ND vaccines in 3 week old chicks found that at 2 weeks post vaccination 100% of the chicks vaccinated had detectable antibodies in their sera. Antibody titres were highest at 2 weeks post vaccination with vaccine from three different sources being 5.6, 3.9, and 4.0 log_2 respectively.

Abdu *et al*. (1999), also found out that the percentage of chicks with NDV antibodies was highest for chicks vaccinated with vaccine from birds given one field dose of the vaccine and birds given ten (10) times the field dose while that for birds given $1/10^{th}$ field dose peaked at three weeks post vaccination. The mean NDV antibody titres of all vaccinated birds in the same experiment were found to decrease to 2.0 log_2 by the 13^{th} week post vaccination and percentage (94.0 – 95.0%) of chicks positive for NDV antibody for the groups were comparable.

Host factors such as age, acquired immunity, availability of concurrent disease, breed of birds and prevailing environmental condition play some role in the epidemiology of ND. Beard and Hanson (1984), showed that chicken become increasing resistant with age. Velogenic NDV strain has been found to cause disease in healthy non-immune adults but with some of the birds surviving. Immunity induced due to previous exposure to NDV may be protective (Martin, 1992). Circulating antibodies, secretory antibody, cell-mediated immunity and non-specific surface immunity have been found to play a significant role (Martin, 1992; Young *et al*., 2002). Protective immunity is generally accepted to correspond to $\log_2$ HI titre of 3 or more (Allan and Gough, 1974; Saglid and Henresnapes, 1987), although some birds with low or undetectable HI antibody titres following vaccination or infection were nevertheless protected against challenge (Beard and Hanson, 1984). The duration of active protective immunity varies with the immune status of the bird at the time of exposure to the immune stimulus, the nature of the immune stimulus which depends on the dose of the virus, the route of infection and the strain of the virus (Ibrahim *et al*., 1981; Westbury, 1984). A virulent NDV V4 strain acquired by contact with orally vaccinated birds provided only transient protective HI titre levels (Samuel and Spradbrow, 1989). Immune birds surviving artificial challenge with a virulent NDV have been shown to maintain a mean $\log_2$ HI titre of 7 for 15 months (Martin, 1992). HI antibodies from natural infection decline after 3–4 months. Vaccine induced protection lasts for 2–12 months depending on the vaccine strain and route of administration (Beard and Hanson, 1984). Antibodies induced by one strain of NDV protect against all strains of the NDV and maternally derived antibody may be present for up to 5 weeks post hatching (Martin, 1992), hence newly hatched chicks are capable of mounting an immune response when challenged (Spradbrow, 1988). Immune birds develop infection when challenged with virulent NDV (Martin, 1992). The presence of

lentogenic NDV infection in some chicken populations may result in constant infections which periodically boost the immunity of all exposed birds (Samuel and Spradbrow, 1989). This immunity is not sufficient to protect against virulent ND challenge (Martin, 1992). Both lentogenic and mesogenic NDV infections could be superimposed in a village population (Martin, 1992).

Escherichia coli endotoxin given intravenously induced plasma antiviral activity in chickens (Amna *et al*., 2001). When the endotoxins were given 3 days before NDV exposure in chickens the virus titres were significantly decreased from a peak of 10^2 to $10^{0.18}$; $10^{2.5}$ to $0^{0.18}$ and $10^{2.5}$ to 10^0 in the spleens, lungs and kidneys respectively at 72 h post innoculation. When the endotoxin was given 24 h after NDV inoculation, the NDV titre significantly increased from $10^{2.0}$ to $10^{3.5}$; $10^{2.5}$ to $10^{4.5}$, 0 to $10^{2.5}$ in the spleen, lungs, kidneys and liver respectively at 72 h after inoculation (Amna *et al*., 2001). Evidence of the presence of non-velogenic infection in village chicken arose from isolation of virus from healthy birds and from HI titres in high proportions of non-vaccinated village birds which would normally not be expected to survive virulent Newcastle disease (Saglid and Henresnapes, 1987; Bell and Mouloudi, 1988; Grundler *et al*., 1988; Bell *et al*., 1990).

Some rural chicken populations have uniformly low HI titres implying that the birds sampled may not have encountered ND repeatedly (Martin, 1992). This could be due to the village chicken being either free of ND or the disease is occurring in an epizootic pattern with mortality rate close to 100% (Martin, 1992). Martin (1992), reported only highly virulent strains in enzootic regions.

Vaccination of poultry throughout the world makes assessment of the geographic distribution of ND difficult. Nevertheless, international recording and reporting of ND is carried out by the Food and Agricultural Organisation (FAO, 1985). Spradbrow (1988), concluded that ND is still widespread in many countries of Asia, Africa, and in the Americas. Only countries of Oceania (Australia, New Zealand, Fiji, Papua New Guinea, etc.) appear to be relatively free from the disease.

Vaccine administered to immune or partially immune birds may not induce protection (Martin, 1992). Chickens are affected by a wide range of bacterial viral and parasitic diseases (Permin and Hansen, 1998). Concurrent infections and heavy burdens of both ecto and endo parasites renders rural chickens more susceptible to ND than commercial ones because commercial chickens are better fed than the rural chickens. Cherdechai (1988), said that in Thailand indigenous rural birds are more resistant to ND than commercial breeds. Higgins and Shortridge (1988), said there is no evidence for differences in susceptibility between local breeds in Hong Kong and smallholding and imported breeds. Newcastle disease occurs more commonly and is most severe during climatic stress as outbreaks are often associated with change of season, especially the beginning of the wet season (Jintana, 1987; Ronohardjo and Dirdja, 1988; Thitisak *et al*., 1988; Martin, 1992). Cold weather exposes chickens to ND outbreaks (Dao Trong Dat and Phamchuc, 1985; Ibrahim and Abdu, 1992), just like the hot weather (Bell *et al*., 1990). Nawathe (1988), found that movement of people and stock during the dry season contributes to the spread of the disease.

2.12 REPRODUCTION IN MALE AVIAN SPECIES

Avian (aves) fish (Pisces) and amphibian species are oviparous and the eggs are fertilized outside the body of the female, whose external genitalia are poorly developed. Their eggs are large and are produced in abundance surrounded by abundant yolk (Etches, 1996).

There are about 6,000 or more species of birds and only a few have been domesticated. Those that have provided food for mankind are distinguished by their high rate of reproduction (Etches, 1996). The function of the male is to produce semen and, in the case of natural mating, to copulate and ejaculate the semen on to the averted cloaca of the female. Sexually active cock produces about 3 billion spermatozoa daily, producing about 35,000 sperm cells per second. This sperm production is carried out by the testes which have both a spermatogenic and an endocrine function (Etches, 1996).

2.12.1 The Testes

The testes are suspended from the dorsal body wall in the midline adjacent to the anterior ends of the kidneys just posterior to the lungs. Spermatogenesis proceeds at the internal body temperature of 41°C as opposed to scrotal temperatures of 24 - 26°C in other mammals (Etches, 1996). The post abdominal airsacs are close to the testes and it was thought that this might reduce the temperature due to air movements of inspiration and expiration but it was suggested that spermatogenesis is more intense during the night when the activity of the fowl was lowest and the body temperature may be reduced as high temperatures interfere with spermatogenesis in birds (Lake, 1971). The supply of blood to and the arrangement of blood vessels within the fowl's testis is simpler than in mammals. Birds have no pampiniform plexus of blood vessels as seen in mammals. The

testicular artery is extremely short and branches inside the testis to form many arterial loops amongst the seminiferous tubules. Venous drainage runs through superficial veins which unite and run directly into the posterior vena cava, the common vein is extremely short. The tunica albuginea of the testis is extremely thin. Interstitial tissue containing blood vessels and leydig cells which secrete androgens is not readily visible in histological section of the testis of a mature cock because of the great expansion of the tubules (Lake, 1971). In the female, only the left ovary is functional but in the male poultry both testes are functional. As sexual maturity is attained, the weight of the paired testes increases from 2–4 g to 25–35 g. The left testis is usually larger than the right (Etches, 1996).

The ductus deferens lacks the thick muscular coat typical of mammals and is the main storage organ for spermatozoa on each side of the abdomen (Lake, 1971). The paired testes of the male fowl, turkey, duck, goose and guinea fowl are situated within the abdomen suspended from the dorsal wall on either side of the midline adjacent to the anterior parts of the kidneys and just posterior to the lungs. Semen is produced in the testes and the ducti deferentes. Semen is stored in the ducti differentes which end in two papillae projecting into the cloaca (vent) in the fowl, guinea fowl and turkey. On massage during semen collection, the papillae become swollen and very prominent in the fowl. The ducti deterentes in the duck and goose open at the base of the penis in the cloaca and semen which exudes from them flows down grooves spirally arranged through the length of the projecting penis. In response to massage in the cock, the tissue in the cloaca which forms part of the copulatory appendages (phallus), is caused to erect and ejaculates (Lake and Stewart, 1978).

2.13 NATURAL MATING BEHAVIOUR

With the exception of pigeons in which bonding of the pair is an essential part of the reproductive process, broilers and layers are polygamous. One male is required for 7–14 females depending on the breed, body condition and age of the flock (Etches, 1996). The physical presence of the male is not required to stimulate ovulation in the female and mating has no apparent effect on ovulation (Lake, 1981). The male : female ratio of broiler breeders is lower than that of layer breeders. The addition of males to an established flock however is associated with aggressive encounters between males as the social hierarchy is re-established (Guhl, 1962). The establishment of a stable social ranking precedes the onset of normal mating activity. Maximum fertility is not obtained until the social order is established (Guhl, 1962; Lake, 1971). High ranking males mate more often than low ranking ones and interfere with the mating performance of low ranking males particularly if the mating occurs in close proximity (Guhl, 1962; Lake, 1984). In large flocks, males establish a territory and attempts by a foreign male to mate within this territory are met with stiff resistance. Mating behaviour in chickens has been evaluated by direct observation (Guhl *et al*., 1945; Crawford and Smith, 1964; Siegel and Caross, 1965) and indirectly based on fertility. Cockerels may mate up to 30 times per day and half of these mating may not be associated with semen transfer. Mating is usually preceded by courtship of females by the male. Behaviours most strongly associated with mating in poultry are:

(1) Waltz; here the male drops one wing and approaches the hen with short shuffling side steps.

(2) The rear approach in which the male holds the comb or neck of the hen or flaps his wings over her. Other courtship activities include circling around the hen with

exaggerated high steps, “tidbitting” in which it pecks and scratches at the ground while giving food calls (Guhl, 1962; Lake, 1984). The cock runs to a corner stamps his feet and settles down in the litter with movement that make a nest-like depression while vocalizing to the hens, this is called “cornering”. Other behaviours like dust bathing, wing flapping, head shaking, feather ruffling, tail-wagging, bill-wiping, preening, strutting and whining vocalization appear to transmit a courtship signal to the hens even though these behaviours are not specifically associated with courtship (Lake, 1984). The males direct most of their courtship behaviour to the entire flock; waltzing and rear approach are clearly directed to individual females. Courtship by the male initiates a selective response from the female who may accept or reject his advances.

The receptive hen crouches before the male with her legs slightly spread. The cock mounts, grasps her neck feather with his beak and treads on the hen’s back, the hen raises her tail and moves it to one side while the male lowers his tail and slides over the rear of the hen. Semen is ejaculated through the engorged phallic, folds (penis) onto the everted cloaca of the female as the two organs make brief contact. The averted cloaca of the hen is retracted immediately as the male dismounts the female. The female then stands up and shakes vigorously while the male may circle or waltz (Guhl, 1962; Lake, 1971). Cloacal contact lasts for less than one second as the semen is transferred from the male to the female.

Courtship by turkey toms is called “strutting”. The male drops his wings such that the tips scrape the ground while stepping forward in a deliberate motion and “trumpeting” a series of deep notes. The frequency of this behaviour is increased as sexual maturity is

attained, “strutting” has been observed in toms just a few weeks old (3–4 weeks). Its frequency can be induced prematurely by giving estrogens (Lake, 1984), when sexually nature males are penned together the toms tend to display “strutting” at the same time and they trumpet in synchrony (Lake and Stewart, 1978).

2.14 SEMEN PRODUCTION

The expression of various physiological traits including semen production can be influenced by certain environmental factors to which the birds are subjected, such as type of housing, staff changes, light, temperature, nutrition, disease, drugs, pesticides, etc. (Lake, 1971). Adequate light intensity and duration is needed for good semen production. Semen quality is a better indication of reproductive performance. Fertilization requires good semen quality such that spermatozoa will be able to reach and penetrate the egg yolk (Mellor, 2001). The most important semen characteristics are sperm motility, viability, concentration, per cent live spermatozoa and total abnormalities.

Reproductive inefficiency is recognized as the costliest and limiting constraint to efficient animal production. Both qualitative and quantitative characteristics of semen have a marked effect on egg fertility (Kamar, 1960; Chalow, 1970). There has been an appreciable amount of work on semen production and quality in a variety of poultry breeds. Semen production is highly correlated with breed body weight and testis size (Burrows and Titus, 1939; Williams and McGibbon, 1943; Boone and Hughes, 1969; Saeid and Alsoudi, 1975). Seasonal variations in semen production have been reported (Wheeler and Andrews, 1943; Polge, 1951; Kamar and Badreldin, 1959 and Saeid and Alsoudi, 1975). Saeid and Alsoudi (1975), reported similarity in semen quality between local Iraqi cocks and some popular imported

breeds. Egbunike and Oluyemi (1979) in a comparative study of Nigerian White Rock, Rhode Island Red, White Leghorn and Nigerian indigenous breed found that the semen volume and motility were highest in White Rock cocks and lowest in the Nigerian indigenous breed, the White Rock had the lowest spermatozoa abnormalities and the Rhode Island Red had the highest abnormalities.

2.15 SEMEN COLLECTION

Apart from inherent physiological factors that influence the quality and production of spermatozoa, there are aspects of the collection and preparation of semen for insemination which have to be taken into consideration so as to achieve high fertility. There are several methods of semen collection in poultry.

With minor modifications between poultry species semen, is commonly collected by massaging the back of the male. This stimulates the erection of the phallic structures and the papillae, which are the terminal portions of the ducti deferentes projecting into the cloacae. The semen is then squeezed into a collecting vessel (Lake and Stewart, 1978; Lake, 1981; King, 1981). Lymphatic folds within the cloacae of the fowl also become everted in response to the massage stimulus and from these folds a variable amount of a transparent fluid is obtained when the copulatory organ is squeezed to obtain the semen. The swelling is much greater generally in broilers than in the layers resulting in more transparent fluid being released by the broiler cock resulting in semen samples with reduced concentration of spermatozoa (Lake and Stewart, 1978). In the drake (ducks) the fluid emanates from the cloacal wall and flows down the penis (Nishiyama *et al*., 1976). The temperament of the male differs between breeds and strains of fowl and

between different types of poultry. This governs the ease with which the male can be captured and handled, which in turn determines to some extent the amount of force needed to express semen. Very excitable males may yield little semen and the tendency for an inexperienced handler to express excess transparent fluid. Transparent fluid is believed to be ejected to some extent with semen from the ductus deferens at natural mating. It causes a rapid loss of fertilizing ability in fowl and duck spermatozoa after prolonged holding *in vitro* (Nishiyama, 1955; Lake, 1956; Nishiyama, 1961; Nishiyama, 1971; Fujihara and Nishiyama, 1976; Lake, 1981, 1983), particularly at high ambient temperatures. To maintain high fertility using artificial insemination, do not collect semen contaminated with excessive amount of transparent fluid. Apart from spermercidal effects, it decreases the density of spermatozoa in semen samples leading to a decrease in fertility. Semen quality is also influenced by fecal and urine contamination in the cloaca during the excitement caused by semen collection. This affects the quality of semen particularly if the contaminated semen is held *in vitro* for too long. The higher the ambient temperature the quicker the detrimental effect on spermatozoa (Lake, 1956). Semen is collected soon after birds have been fed and watered because the chances of excretion on massage are enhanced, thus contaminating the sample.

2.15.1 *Semen collection method in the domestic fowl (cock)*

In the domestic fowl, semen is usually obtained by gently massaging (stroking) the back two or three times with the palm of the hand. The hard stroke over the back ends towards the tail with the other hand. The hand stroke over the back ends with the thumb and forefinger making a slight upwards movement at the base of the tail. The legs can be felt to stiffen and the tail rises with a good response. Two persons can also perform

semen collection, one holding the male by the thighs and the other massaging and collecting the semen in a collecting tube. Erection of the copulatory appendages in the cloaca is caused by these stroking movements and when this occurs the semen must be immediately expressed from the swollen ejaculatory papillae (ducts) by gently pressing inwards on either side of the cloaca with the thumb and forefinger of the hand that was used for massage. The bulk of the spermatozoa are obtained in the first portion of the ejaculate. If the ejaculating appendages are squeezed too hard blood will appear in the semen due to the rupture of the vascular tissue (Lake and Stewart, 1978).

2.15.2 *Semen collection method in the turkey tom*

Collection of semen in the turkey tom is more prolonged than in the male fowl, due to its size and body weight. The turkey tom should be picked up without struggling so as to get the best response during semen collection. The two persons approach is used whereby one person holds the bird's legs and operates the semen collection apparatus while the second person holds the tom and massages the area around the cloaca. If three persons are involved in the semen collection the one who applies the massage sits astride a rectangular platform or bale of straw. The male is placed on its chest in a vertical position with its neck under one thigh of the masseur and its legs over the masseurs other thigh. The legs of the bird are held firmly by the third person. If the turkey tom is facing the left of the masseur, his left hand is placed on the back of the turkey tom about 15 cm in front of the tail. A firm stroking motion towards the tail is made once or twice to help elicit the erection of the copulatory appendage (phallus) in the cloaca. Each tailward stroking motion should end by sliding the thumb and forefingers around the fleshy portion of the tail. After a few strokes both hands of the masseur massage the broad area around the

side of the pubic bones whilst the left forearm presses the tail onto the back of the bird exposing cloaca maximally. Care must be taken not to rub too hard during massage otherwise bruising and rupture of the tense skin around the cloaca may occur. The erectile copulatory appendage should protrude from the cloaca after several gentle massage movements. The copulatory appendage in the turkey tom is larger than in the male fowl and rises up prominently from the floor of the cloaca. In trained toms this response occurs after massage for only a few seconds. Using the thumb and forefinger of the left hand, pressure should be applied deep at either side of the cloaca at the instance when the copulatory appendage becomes fully protruded. Continuous pressure is exerted to squeeze the semen from the bulbous ducts (papillae) which are hidden below the surface at the base of the erectile tissue. Mechanical methods have been devised to make semen collection in turkeys easy. The male is strapped to a collecting chair, one person is in charge of massaging the male turkey and collecting the semen into a vessel with a hand suction gun. The frequency of semen collection in turkey toms is as in the male fowl (Lake and Stewart, 1978).

2.15.3 *Semen collection method in water fowls (drake and gander)*

Male waterfowls (drakes and ganders) respond to massage as the cocks and toms. The drake and gander have a coiled penis-like appendage which, before mating, erects and unwinds from the cloaca in response to massage (Lake, 1971). In the duck one method of semen collection involves a person sitting on a stool with the drake squatting on the lap with its head pointing to the left. The left hand strokes the back of the bird and the right hand strokes the abdomen towards the cloaca. Alternatively, the right hand holds the base of the wings which helps to hold down the bird on the lap. After several stroking

motions the thumb and the forefinger of the right hand lightly massages the area around the pubic bones. This may cause the extrusion of the penis and ejaculation; the semen flows down the spiral-shaped penis after firm pressure has been applied around the cloacal region and semen can be collected. The time required for inducing ejaculation in palmiped birds is about 30 seconds, some will respond in 10 seconds (Lake and Stewart, 1978).

The principle of collecting semen from ganders is similar to that of the drake, because of their large size they are placed on the padded top of a trestle stool before massage. One person stands facing towards the rear of the gander holding the legs on either side of the stool and the neck of the gander under the right arm. The other person strokes the back of the gander with the right hand towards the vent as for the drake (Lake and Stewart, 1978).

2.15.4 *Semen collection method in the guinea fowl*

In the guinea fowl the male is laid resting on its breast on the knees of a seated person with the cloaca raised upwards by slightly tilting one knee while a second person holds the legs in one hand and collects the semen (Lake and Stewart, 1978). To elicit erection of the copulatory appendage, the seated person massages the back to the base of the tail with one hand and gently massages the abdomen around the cloaca with the other hand. The erectile organ is longer than that of the male domestic fowl and consists of elongated bulbous folds with a groove between (Lake and Stewart, 1978). With pressure from the thumb and forefinger the semen flows from the tip and should be quickly aspirated into a collecting apparatus similar but smaller than that used for the male turkey.

2.15.5 *Artificial vagina*

Semen can also be collected using an artificial vagina (Fomin and Shcherbatov, 1989). A dummy with an artificial vagina is used to collect ejaculate after teasing with hand held hen for three minutes prior to substituting a dummy for live hen. The training period for cocks to get used to dummy is about 3 days.

2.16 SEMEN EVALUATION AND DILUTION

As soon as semen is ejected from the male a proportion of the spermatozoa begin to lose their integrity and this continues during storage *in vitro* leading to a decline in fertility after insemination (Lake, 1983). After collection the container containing the semen is placed in a water bath at about 40°C. The evaluation of each ejaculate usually include gross or visual evaluation soon after collection in respect of volume, colour and gross sperm motility (microscopic examination of wave pattern) as described by Zemjanis (1970).

Gross sperm motility is usually determined by examining a drop of raw undilated semen on a pre-warmed slide under a light microscope at X 100 magnification. The live dead counts and sperm head morphology are evaluated by using the eosin-nigrosin vital staining techniques. Samples fixed in buffered normal saline are used for evaluating cell morphology (spermatozoa abnormalities). Semen concentration is usually determined by the use of hemocytometer. Research on diluents and storage techniques is aimed at providing the best possible conditions for their maintenance (Lake, 1962). If spermatozoa are to be stored beyond an hour they must be suspended in a synthetic diluent, careful handling being essential before and during the process of dilution to minimize the loss of viable spermatozoa. Significant improvement in fertility and hatchability in turkey semen

extended with Lake's diluent containing gentamycin has been reported by Omprakash *et al.* (2006). Moderate dilution (1 part of semen to 5 parts of extender) prolongs spermatozoa viability during *in vitro* storage. Excessive dilution of semen more than 1 ml of semen to 10 ml of extender reduces the concentration of the spermatozoa per ml, this can lead to infertility (Lake, 1967). Excessive dilution (the classic dilution effect) stimulates sperm motility and metabolic activity, which shortens the life span of spermatozoa *in vitro*. To minimize this, the semen dilution ratio should be 1:1 or 1:5 during storage of spermatozoa of both chicken and turkey semen (Lake, 1962). Seminal plasma is deleterious to storage of turkey spermatozoa at 4°C and is involved in phospholipid metabolism of spermatozoa (Douard *et al*., 2005).

2.17 LIQUID SEMEN PRESERVATION

Turkey semen already diluted can be kept between 10 and 15°C for a few hours. A new turkey semen extender called "turkey semen extend" had motile turkey spermatozoa 20 hours after collection and extension of the semen (Morell *et al*., 2005). Lipid peroxidation is a significant factor affecting the fertility of stored turkey semen. Insemination with stored turkey semen yielded lower fertility rates than the control fresh semen in spite of the presence of vitamin E in the stored semen (Long and Kramer, 2003). Cock semen in contrast can be diluted and stored at 5°C for 24 to 48 hours without impairing its fertilizing ability. Chaudhuri and Lake (1988), developed a new method of holding and storing fowl semen at 37°C - 40°C for up to 16 h. This is of great advantage for breeders in the tropics where ambient temperatures are high and refrigeration facilities are rarely available especially under farm conditions. It is known that the pH, osmotic pressure and glucose availability allow sufficient metabolism to occur to maintain cell membrane

integrity and energy to sustain the fertilizing ability of the spermatozoa. Common constituents of poultry semen extenders such as Tris (tromethamine), sodium hydroxide, glucose, sodium chloride and antibiotics are shown in Table 2.1 (Sexton, 1976).

Sexton (1975), developed a new diluent for chicken semen called Beltsvillie Poultry Semen Extender (BPSE) for short time preservation of chicken semen. In this experiment the semen from Leghorn males diluted at 1:4 ratio with BPSE can be stored for up to 24 hours without any critical loss of viability. A number of synthetic solutions that extend as well as aid in semen preservation were formulated and tested by researchers (Sexton, 1978). A good extender should have the following qualities: exogenous energy source; proper osmotic balance; sufficient buffering capacity, control of bacterial growth and ability to protect the semen against toxic ions, i.e. chelating action (Ogasawara and Ernest, 1970). Glutamate, Tris and potassium phosphate have been found to be critical to the survival of chicken semen during storage at 5°C (Sexton and Fewlass, 1978). Stephens (1986) observed that Tris buffer was superior to sodium citrate diluent for preserving cockerels semen. However, Omprakash *et al*. (1992), found that dilution with Tris buffer reduced fertility but not hatchability.

2.18 PRESERVATION OF SEMEN AT SUB-ZERO TEMPERATURES

Poultry semen has been preserved at freezing temperature of between –79°C and -196°C (Watanabe, 1972). The length of time that poultry semen can be frozen and still retain its fertilizing ability has not been firmly established. The longest time chicken semen has been kept frozen is five years, with little reduction in fertility (Watanabe,

Table 2.1 Constituents of poultry semen extenders

Constituents	Primary function
Phosphates (Na+ or K+), TRIS	Buffer
Fructose, glucose, inositol raffinose	Energy source
Glutamate, albumen, milk	Chelating agent
Magnesium chloride, sodium acetate, potassium citrate and sodium chloride	Osmotic balance
Gentamycin, penicillin, streptomycin	Antibiotics

Source: Sexton (1976).

1972; Watanabe and Terada, 1976). The longest reported storage period for frozen turkey semen has been four years, a significant reduction in fertility was seen (Ogasawara, 1977). Reduced fertility reported with frozen – thawed chicken and turkey semen appears to be due to a reduction in a survival of spermatozoa as well as insufficient number of viable spermatozoa reaching the site of fertilization. Vaginal douching with gentamycin reduces microbial load of the vagina and leads to improvement in fertility and hatchability and corresponding reduction in embryonic mortality. Hens inseminated with semen extended with Bellsville poultry semen extender diluent along with vaginal douching have been found to show higher per cent fertility and per cent hatchability (Omprakash and Venkatesh, 2006). Preservation of poultry semen at sub-zero temperatures requires the addition of cryoprotective compounds such as glycerol, dimethylsulfoxide (DMSO) or ethylene glycol (Bacon *et al*., 1985). Semen freezing is more effective in chickens and geese than in most other domestic birds (Blesbois *et al.,* 2005). Although glycerol is used as a cryoprotectant to protect chicken and turkey spermatozoa, subsequent fertility trials showed that spermatozoa in a diluent containing glycerol lost virtually all fertilizing capacity before freezing (Neville *et al*., 1971). To reduce the deleterious effects of glycerol, the concentration of glycerol is diluted from the cryoprotective level of 7% to less than 2% by dialysis or centrifugation before insemination (Lake and Stewart, 1978 and Lake, 1981).

2.19 ACTORS AFFECTING FERTILITY AFTER INSEMINATION

Spermatozoa are able to live in the oviduct of the fowl, geese and ducks for 5 to 11 days and turkeys for 4 to 6 weeks and during these periods fertile eggs are being laid (Wishart, 1996). Some hens lay eggs for a shorter period and some for a longer period so that in

any given period there will be variation in the proportion of fertile eggs laid by each hen (Wishart, 1996). The success of artificial insemination in poultry depends primarily upon the suitability of the semen diluents used and minimizing contamination by bacteria during collection and insemination. Artificial insemination using raw semen is not profitable in chickens (except for experimental purposes) diluents are used for commercial purposes (Das *et al*., 2004). The criteria of success in artificial insemination is the production of the pattern of prolonged fertility in each hen between insemination (Lake, 1969; Lake and Steward, 1978). The environment in the oviduct is most favourable for spermatozoa when the female is in good laying condition. The most intense layers are the most fertile females. Individual birds at or around the peak of lay produce the most fertile eggs (Lake, 1983). The oviduct is most receptive to semen just after an egg is laid and it is recommended that for the best fertility, inseminations should be performed at the time of day when most hens are without hard shelled eggs in the shell gland (Lake, 1969). The spermatozoa by a process yet unknown travel to the top of the oviduct to a region where smaller numbers of spermatozoa are stored and where fertilization takes place. This part of the journey is probably done daily when the oviduct is empty, i.e. between an egg being laid and the next ovulation (Bobr, 1962; Lake, 1969). It is important that an optimum number of spermatozoa enter the primary storage glands at the utero-vaginal junction soon after insemination if maximum fertility is to be obtained. Hence the importance of a good eversion of the vagina and a deep insemination as close as possible to the storage area. Bobr (1962), Lake (1969) and Brillard and Bakst (1990), estimated the total number of spermatozoa within the tubules. They found that only about 1 – 2 million spermatozoa were stored in the tubules of chickens or turkeys after

100 – 200 million spermatozoa were inseminated artificially into the vagina. The majority of the spermatozoa were destroyed in the vagina by phagocytosis. The physiological status of the hen at the onset of the reproductive season can play a major role on subsequent performances of fertility. Brillard and Bakst (1990), demonstrated that hens inseminated just prior to the onset of the laying season store higher populations of sperm compared to hens inseminated just after the onset of lay. The precise mechanism by which spermatozoa are released from the storage tubules in the uterovaginal (UV) region of the hen's oviduct for the fertilization of successive daily ova remained unknown (Burke *et al*., 1972). Spermatozoa introduced into the region either by copulation or artificial insemination when examined microspically in histological tissue appeared tightly packed and arranged in parallel bundles with the tubules with their heads directed toward the blind terminal tubular end (Bobr *et al*., 1965).

The initial quantity and quality of spermatozoa at the time or insemination play a major role in fertility performance and these factors directly influence the degree of selection exerted by the hen on subsequent sperm populations stored in the oviduct (Brillard, 2003). Following a single insemination a maximum of approximately 1% of the initial population of spermatozoa deposited intravaginally reaches the storage sites both in the chicken and turkey hen (Brillard and Bakst, 1990; Brillard, 1993). The quantity of spermatozoa present in the infundibulum at the time of fertilization, that is during the 8 – 9 minutes following ovulation, can be estimated in freshly laid eggs (Brillard and Antoine, 1990). About 80 to 100 million spermatozoa per insemination are necessary to maintain high fertility and that there is no advantage of inseminating more than 100 million spermatozoa at any one time (Lake, 1983). The insemination of 0.025 to 0.05 ml

semen twice weekly or every 4 or 5 days is preferable for maintaining constant high fertility in poultry species (McCartney, 1976; Fiser and Chambers, 1981; Lake, 1983). Transparent fluid is a variable component of semen and a little amount of it does not harm the spermatozoa provided at least 100 million spermatozoa are inseminated (Nishiyama *et al*., 1971; van Wambeke, 1976). Optimal turkey and fowl fertility may be achieved by weekly insemination of about 50 million spermatozoa, this is only likely to be achieved for that part of the breeding period when reproductive activity is at its peak, e.g. semen production and egg production. The dose of 80 to 100 million spermatozoa is more suitable as a constant recommended dose to cover all parts of the period (Tangja and Gowe, 1961; Sexton, 1977). Wall and Jones (1977) indicated that in the turkey after a single insemination for a 3-week period, the number of spermatozoa required for optimum fertility was dependent upon the number of eggs laid. The frequency of insemination and/or the number of spermatozoa (dosage of semen) inseminated may be increased from the middle to the end of the breeding period in an attempt to overcome a tendency for a drop in fertility at that time. Often a change in reproductive capacity occurs in the female at the same time as in the male. Sexton (1977), showed that increased inseminations did not improve turkey fertility in the latter stages of egg production. Bielinska *et al*. (1976), investigated the effect of intervals of 6, 9, 12 and 15 days between inseminations on goose fertility and found that high fertility levels were obtained by adjusting the frequency of insemination according to the age of the ganders. Since the yearlings inherently produce less fertile semen than older ganders, the best interval between inseminations was found to be 6 – 9 days and 12 days, respectively. Borys *et al*. (1978), obtained optimum fertility in geese by inseminating 20 million spermatozoa at 6-day intervals. When spermatozoa are stored *in vitro* before insemination, depending upon the condition of storage, a proportion of them undergo

degeneration, thus higher absolute numbers of spermatozoa must be inseminated to compensate for the loss. van Wambeke (1978), estimated that about 200 million spermatozoa are required to produce high fertility with fowl semen stored for 6 – 24 h at 2°C - 5°C. About 50% of spermatozoa are destroyed during freezing necessitating the insemination of higher doses of thawed semen.

2.20 Timing of Insemination and Deposition of Semen

The distal portion of the vagina must be well averted before semen is inserted into the oviduct and the ease with which this is done varies among species of domestic birds, being most difficult in some water fowls (Lake, 1967). The procedure is carried out by first putting pressure on the abdomen of the female, and then releasing it allowing the oviduct to resume a normal position before actually ejecting the semen in the cannula or syringe so as to deposit spermatozoa into the uterus or shell gland (Lake and Stewart, 1978).

Inseminations should be done when no hard shelled egg is likely to be present in the uterus or at least not within 3 h of the oviposition (Bornstein *et al*., 1960; Parker and Arscott, 1971; Tanaka and Okano, 1971; Lee, 1973; Christensen and Johnson, 1975; 1977; 1978; Giessen *et al*., 1980). Rough treatment of hens must be avoided during capture before insemination and each female must be released gently after insemination otherwise semen may be expelled from the vagina. Macpherson *et al*. (1977), reported a lowered fertility in hens which were thrown down on to litter after insemination. Meyer *et al*. (1980), suggested that turkey hens might be disturbed by too frequent handling especially towards the end of the breeding period. They found that weekly handling of the birds for artificial insemination caused a drop in egg production and overall fertility.

Stress may interfere with spermatozoa transport in the oviduct with consequent effect on the fertilization rate. Kurbatov *et al*. (1976), found out that fertility was lowered if semen was deposited too deeply in the vagina in geese. Spermatozoa deposited within 1–3 h prior to or just after oviposition are eliminated by the vaginal contractions involved in the process of oviposition (Brillard *et al*., 1987; Brillard and Bakst, 1990). From a practical stand point the period of insemination in a given breeder house should be carefully scheduled in order to minimize the risks of performing a majority of inseminations at or around the time of lay. Chicken breeder hens at peak lay most of their eggs between the 5^{th} and 8^{th} hour following the onset of the photoperiod (16L: 8D), i.e. sixteen hours light and eight hours darkness (Brillard, 2003). Breeder turkey hens at peak would lay mainly from the 7^{th} to the 9^{th} hour of lay (14L: 10D). In both species the laying period is delayed by 2 hours in aging flocks (Brillard, 2003). It is the sperm in the storage tubules which support fertility during the ensuing days or weeks. Spermatozoa after being released from the tubules are transported upwards quickly towards the infundibulum where the ovum is normally fertilized (Wishart, 1996). Spermatozoa disappear at the rate of 29% in the chicken and 9% in turkeys each day following insemination or natural service in the female. The hen loses oviducal spermatozoa more quickly than the turkey as a result the number of spermatozoa which reach the egg fall below the threshold which ensures fertility sooner and this explains why the length of the fertile period is longer in the turkey than the hen (Wishart, 1996).

Spermatozoa draw nutrients from two main sources, the semen plasma and secretion of the female genital tract during natural service (Mann, 1948). Regardless of the diluents used pre-insemination douching with ampicillin solution reduces the microbial load in

and around the vagina leading to better spermatozoa survival which leads to increased fertility (Omprakash *et al*., 1992). Gale and Brown (1961), on the contrary noted that most antibiotics when used at the concentration necessary for antibacterial activity were spermicidal. Hens treated with Pregnant Mare Serum Gonadotropin (PMSG) which contains mainly FSH activity had a lower rate of release of spermatozoa from the uterovagina sperm storage tubules and a decreased rate of transport of spermatozoa up the oviduct (Zavaleta and Ogasawara, 1987).

2.21 AVIAN REPRODUCTIVE PHYSIOLOGY

Fertility levels after insemination may be influenced by the effect of oviducal environment on the transport of spermatozoa and the retention of their fertilizing ability. Howarth and Huston (1974) showed that extract of oviducts of fowl hens kept at 31°C had a greater stimulatory effect on glucose utilization by spermatozoa than those kept at 19°C, thus a decline in fertility of hens exposed to high environmental temperatures could be partly due to a defective oviducal environment affecting the metabolic activity of spermatozoa. Fertility declines with age or season according to egg productivity, broilers and turkeys fertility declining sooner than layers. Ogasawara and Funqua (1972) and Christensen (1981), found few spermatozoa in the UV glands in the turkey hen during the seasonal drop in fertility implying that the conditions of the oviduct had changed and it was unable to sustain many spermatozoa. This view was supported by Sexton (1977), who was not able to alleviate a late season decline in turkey fertility by multiple inseminations. Yu and Burke (1979), showed that naturally low fertility in turkey hens could be improved by intra-magnal insemination. Fertilization of eggs by aged spermatozoa affects fertility

and hatchability because the aged spermatozoa cannot pierce through the eggs to fertilize the ova (Lodge *et al*., 1971; Lodge *et al*., 1974; Popescu and Merat, 1977). Fiser and Chambers (1981) found that if the interval between inseminations is extended beyond seven days the hatchability of eggs set is reduced due to a greater incidence of infertile eggs and a gradual increase in early embryonic mortality. However, van Krey and Siegel (1976), did not consider aged spermatozoa to be the cause of embryonic death with insemination at 7-day intervals.

2.22 IMMUNITY AGAINST SPERMATOZOA

Fertility levels in fowl and turkeys bred by artificial insemination might be affected by hens generating antibodies against spermatozoa rendering them ineffective in the oviduct (Lake, 1969). The phenomenon could be the cause of drop in fertility in these birds, though there are other causes such as disease, nutrition, or faulty artificial insemination technique. Choi (1976) injected whole fowl semen intravenously, intramuscularly and intravaginally into hens and produced raised titers of sperm agglutinating antibodies in the blood plasma. Very high titers were accompanied by lowered fertility and intravaginal inseminations resulting in an increase in antibodies in the blood. Burke and Yu (1979) and Yu and Burke (1979) also found that antibodies to spermatozoa can impair the function of spermatozoa *in vitro*.

2.23 FACTORS AFFECTING REPRODUCTION IN MALE POULTRY SPECIES

2.23.1 *Age and breed*

Semen production starts at about 20 weeks in cocks and stabilizes after 35 to 36 weeks (Culbert *et al*., 1977). Schrocksnadel *et al*. (1971), noted increased testosterone levels as the male chicken approached maturity. Libido in the male is not necessarily correlated with high fertility, the most frequent copulation produce many aspermic matings (Schrocksnadel *et al*., 1971). The reproductive capacity of the testes and genital organs to produce semen is influenced by age and breed (Schrocksnadel *et al*., 1971).

Munro (1938) noted that survival of spermatozoa in the ductus deferens is not androgen dependent, but the phallus and vascular bodies structures associated with the transparent fluid formation appear to be androgen dependent (Nishiyama, 1955). Commercial egg-laying male strains of fowl produce small amounts of semen between 16 to 20 weeks of age, but good semen for artificial insemination purposes may not be obtained until when the cocks are 24 to 32 weeks of age under good management (Culbert *et al*., 1977). The age of onset of semen production and the maximum volume of semen obtainable from a male bird depends to some extent upon the breed and strain, age of sexual maturity, the season and the lighting schedules during rearing (Etches, 1996; Lake and Stewart, 1978). Medium weight laying strain and broiler male generally mature latest and they yield semen at between 26 – 28 weeks of age on a constant 14 h day length (Brillard, 2003).

Turkey toms begin to produce semen when 21 weeks old but sufficient semen of good quality is obtained at 32 to 36 weeks of age. The most common morphological

defects of turkey's semen are acrosomal and midpiece defects (Alkan *et al*., 2002). Drakes will give semen when 24 to 28 weeks old, while the gander and male guinea fowl of the same age will produce semen at 26 to 28 weeks of age. With exceptionally good management the male guinea fowl can start producing semen at 5 to 5.5 months of age (Lake and Stewart, 1978). The volume of semen obtained from various types of poultry is variable depending upon the species, breed and strain of the bird, age and nutrition (Lake and Stewart, 1978).

2.23.2 *Temperature*

Ambient temperatures may influence reproductive ability by altering the food intake of the bird. There may be breed differences in adaptation to temperature, thus varying the response to changes in temperature (Lake, 1971). Low ambient temperatures of about 8°C were found to retard both testis growth and spermatogenesis in growing white Plymouth Rock males. High ambient temperature of about 40°C interferred with spermatogenesis in mature male Plymouth Rock cocks (Lake, 1971). Follicle stimulating hormone (FSH) secretion in the hen's pituitary is influenced possibly by temperature (Lake, 1971).

2.23.3 *Photoperiod*

Light is an extremely important factor stimulating gonadal activity in birds, a r*etino-hypothalamo-hypohyseal pathway* is involved (Benoit, 1964). The body and gonadal growth is invariably influenced by light of different wavelengths. Pituitary and testis weights tended to be greater in male chicks reared under violet-blue or red light for about 21 days (Benoit, 1964). Various effects on growth and reproductive performance of poultry can be produced by manipulating the daily photoperiod light quality and light

intensity. Light treatments can influence activity and food intake in the bird thereby affecting growth and reproduction (Lake, 1971). Exposure of birds to 14 h light daily hastened the onset of semen production (Lake, 1971). Most hens will commence egg production by 24 weeks of age when exposed to 8 h light and 16 h of darkness throughout their life (Etches, 1996). The age at sexual maturity for these hens can be advanced to 22 weeks by providing photo stimulation. Exposure of castrates to short days produces a slow rise in plasma Luteinising Hormone (LH) and a maximum LH level that is much lower than that observed when castrates are transferred to long days. The rate of increase in plasma LH following castration is greater in capons exposed to long days (Etches, 1996). When birds have been exposed to long days for an extensive period exposure to short days intensifies inhibition of GnRH neurons and reproduction ceases (Etches, 1993).

2.23.4 *Dirnal rhythm*

Greater numbers of spermatozoa were obtained in collections made between 6 pm and 7 pm than in the morning or at mid-day (Lake, 1971). It is not known whether this phenomenon is associated with rhythmic changes in the metabolic rate. Spermatogenesis varies with possible diurnal changes in body temperature. The period of highest mating activity was found to coincide with that of greatest semen yield (Lake, 1971). Rekwot *et al*. (1997) found that testosterone peaks in bulls occurred mostly in the morning hours and ranged from 1 to 5 peaks in number.

2.23.5 *Genetics*

Semen production in cocks is believed to be inherited (Lake, 1969). Heritability values were found to be high in semen of white Rock cocks for motility and concentration of spermatozoa and volume. Rate of body weight gain was negatively correlated with motility and there was slight positive correlation between motility and fertilization rate in natural mating (Lake, 1971), Nwagu *et al*. (1996) working with Rhode Island Red and white cocks found that the Rhode Island white cocks had better spermatozoa concentration than the Rhode Island Red cocks. The Rhode Island White cocks produced lower semen volume and better color than the Rhode Island Red cocks.

2.23.6 *Nutrition*

Adequate food is essential to maintain males in good reproductive condition (Boone *et al*., 1967). Starvation for 6 days leads to a marked reduction in semen production (Boone and Hughes, 1969). Males are often obliged to consume food intended for laying hens and studies on specific nutrient requirements for semen production are rare (Boone and Hughes, 1969). The effect of dietary calcium levels on semen characteristics of white leghorn males were studied. Semen characteristics and fertility differed insignificantly hence semen characteristics and fertility were not affected by nutrition (Lake, 1971). Dietary protein during growth of males influences the onset of sexual maturity, 9% crude protein or less delays sexual maturity (Lake, 1971). Adult White Leghorn males fed 9% dietary protein produced normal quantities of semen. This 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 counterparts. Gallo *et al*. (2005) found that selenium supplementation is a crucial factor in maintaining high reproductive characteristics of broiler breeder males.

One of the most important problems of extensive poultry production is the unsteady nature of feed supply and availability resulting in undernourished birds. It is of interest therefore, to simulate the feeding situation under the extensive production system to assess how this may affect semen production and quality in Nigerian local cocks. Ezekwe *et al*. (2003), found that the effects of underfeeding local cocks on 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 testis are more responsive to underfeeding than the secretory activity of the reproductive tract. Underfeeding diminishes the ability of the gonads to respond to gonadotropin stimulation (Davies *et al*., 1957). Moderate underfeeding and reduction of feed intake by 30% to 50% will not adversely affect semen production and quality of local cocks (Ezekwe *et al*., 2003). It is consdered 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 (Ezekwe *et al*., 2003). It has been reported that protein intake hastened the onset of puberty in heifers and bulls (Oyedipe *et al*., 1981; Rekwot *et al*., 1987a).

2.23.7 *Season*

There is abundant literature on the influence of season on the reproduction of domestic animals (Rekwot *et al*.,1987b). Ejaculate volume, sperm concentration and

percentage live spermatozoa of bulls were significantly higher in the rainy season than the dry season (Rekwot *et al*., 1987b; Sekoni *et al.,* 1988b).

It is concluded from the results of the above studies that fertility in bulls is higher in the rainy season in Nigeria than in the dry season. The implication here is that semen should be collected and preserved in the rainy season for artificial insemination and that matings should be encouraged in the rainy season. High ambient temperatures interfere with spermatogenesis in the cock (Lake, 1971). Semen production is influenced by light, temperature and nutrition (Lake, 1971). Low ambient temperatures and light of adequate intensity and duration are found in the rainy season months. It will be expected that good quality semen will be produced at this time. Onuora (1982) found that guinea fowls produce good quality semen in cooler periods of the year.

2.24 REPRODUCTIVE DISEASES OF POULTRY

There are many diseases affecting egg production and quality in hens with limited and scanty information on the diseases affecting semen quality in their male counterparts. Egg production is a sensitive indication of health in the female fowl and any factor causing ill health or sudden change in fitness will adversely affect egg production (Jordan, 1990).

2.24.1 *Mycoplasma infections*

Mycoplasma infections affect both chickens and turkeys with resultant decrease in hatchability, drop in egg production and salpingitis in chickens (Jordan, 1990). Mycoplasma infections have been found in the semen of turkey toms. Treatment

with enterofloxacin and tylosin eliminated the organism from the semen (Tomczyk and Minta, 2002). *Mycoplasma iowae* infection causes reduced hatchability in turkey hens. The organism is transmitted through the eggs which probably become infected in the oviduct where the mycoplasma is mainly found in the adult female. Venereal transmission is of considerable importance in initiating and maintaining infection of the oviduct. The male is significant in the transmission of this infection. The disease can be spread through artificial insemination using infected semen (Jordan, 1990).

2.24.2 *Egg drop syndrome (EDS)*

EDS 76 is caused by an *Adenovirus*. The virus differs from conventional adenoviruses in that it agglutinates avian but not mammalian erythrocytes to high titres (Jordan, 1990). It is a natural adenovirus of ducks and geese which infect the fowl. It grows best in duck and geese cells and also in chick embryo liver and chick kidney cells (Jordan, 1990). It grows to high titres in embryonated duck eggs but poorly in fowl embryos (Nawathe and Abegunde, 1980; Jordan, 1990). Eight days after infection there is massive growth in the pouch shell gland region of the oviduct coincidental with the production of egg shell changes. Both normal and affected eggs contain the virus externally and internally for two weeks. Chicks hatching from infected eggs often do not develop antibody but they are latently infected (Jordan, 1990). The virus is reactivated at around peak egg production and the cycle recommences. The virus spreads when introduced into primary breeding stock, probably through a vaccine grown in duck cell. Lateral spread also occurs between flocks due to contaminated eggs, contaminated needles and possibly biting insects (Jordan, 1990). The first signs are usually loss of shell strength and pigmentation, followed by thin shelled, soft shelled and shell-less eggs (Jordan, 1990). Classical EDS is manifested by a sudden fall in egg production. It is not known whether affected males

harbour the virus in the semen. If this is proven then the disease can be spread through natural mating and artificial insemination (Jordan, 1990).

2.24.3 *Eastern Equine Encephalitis virus and highlands J. virus infection*

The genus alphavirus includes 27 viruses, the best known being Eastern Equine Encephalitis virus (EEEV), Western Equine Encephalitis virus (WEEV) Venezuelan Equine Encephalitis virus (VEEV) and Highlands J. virus (Guy *et al*., 1995). During the summer and fall of 1991 several turkey breeders experienced acute drops in egg production (Wages *et al*., 1993). Eastern Equine Encephalitis virus and HJ virus were identified as the causes of the decreased egg production in the turkey hens. This was based on isolation of the viruses from turkey hens experiencing drops in egg production (Guy *et al*., 1995; Wages *et al*. (1993). Decreased egg production was experimentally produced by infecting breeder turkey hens with Eastern Equine Encephalitis virus or Highland J virus (Guy *et al*., 1995). These viruses were shed in the semen of turkey toms infected with the virus experimentally (Guy *et al*., 1995). This proved that these viruses are transmitted sexually and artificial insemination of turkey breeder hens is a potential mechanism for transmission of these viruses (Guy *et al*., 1995).

2.24.4 *Western Equine Encephalitis virus infection*

Western Equine Encephalomyelitis (WEE) is an arthropod-borne viral disease primarily affecting equines and humans (Chamberlain, 1987). The disease is prevalent in the western and central United States of America (USA), Canada, Mexico and South America (Walton, 1992). Mosquitoes serve as the primary vectors and wild birds are the reservoirs (Chamberlain, 1987). Culex mosquitoes can infect chickens pheasants and blackbirds this is seen in the San Joaquin and Sacramento valleys in California

USA (Hardy, 1987). Evidence of frequent infection with the virus has been found in house sparrows, house finch, blackbird and domestic chicken in California (Chamberlain, 1987). Serologic surveys of wild turkeys in Texas indicated that the disease is common in them (Trainer *et al*., 1968). It was discovered that egg production drop was accompanied by an increase in semen antibody titres to WEE between acute and convalesant sera in turkeys from affected flocks (Cooper *et al*., 1997). In an experiment carried out by Cooper and Medina (1999), there was no mortality or obvious sign of illness observed. The infection resulted in mild lymphoid necrosis in the thymic gland and bursa of Fabricius and produced a viremia that lasted up to 3 days post inoculation.

CHAPTER 3

EFFECTS OF A VELOGENIC NEWCASTLE DISEASE VIRUS ON PACKED CELL VOLUME, TOTAL PROTEIN AND HEMAGGLUTINATION INHIBITION ANTIBODY TITRES OF VACCINATED SHIKABROWN COCKS

3.1 ABSTRACT

Fifty 20 week old Shikabrown cocks consisting of 22 red Shikabrown and 28 white Shikabrown cocks were purchased from the National Animal Production Research Institute, Shika and used for this study.

Twenty-five of the cocks made up of 8 red and 17 white cocks selected on basis of weight were infected with 2 ml of $10^{6.0}$ $EID_{5.0}$ of a Velogenic Kudu 113 strain of Newcastle disease virus intranasally and orally. The remaining twenty-five cocks made up of 14 red and 11 white served as control. Using heparinized capillary tubes blood samples were taken from the wing veins of both infected and control cocks and centrifuged in a Hermle Z364 centrifuge at 251.6 g for 15 minutes. The centrifuged contents of the capillary tubes (the packed RBCs and plasma) were used to evaluate the packed cell volume and total protein of the cocks. Two mls of blood were taken from the wing vein of each control and infected cock allowed to clot and centrifuged in a Hermle Z364 centrifuge at 251.6 g for 15 minutes, the sera obtained were used to determine Newcastle disease antibody titres.

There was no significant difference in the packed cell volume of the control and infected red Shikabrown cocks. The infected cocks, however had slightly lower values. Similarly there was no significant difference in the packed cell volume of the control and infected white Shikabrown cocks, although the infected cocks had slightly lower values.

Statistical analysis of the values of the total protein did not show any significant difference between the control and infected red cocks and between the control and infected white cocks. The antibody titres of the control red and white cocks were significantly ($P<0.05$) lower than those of the infected red and white cocks.

The finding above showed that the challenged red and white cocks had high antibody titres and a slight drop in packed cell volume. The mean antibody titres of 1.9 ± 0.7 to 4.6 ± 0.4 $\log_2$ provided protection to the Shikabrown cocks against the velogenic Newcastle disease virus since none of the challenged cocks died. In an endemic environment like Zaria poultry farmers keeping Shikabrown chickens should vaccinate them against Newcastle disease. Challenging the red and white Shikabrown cocks with the velogenic Newcastle disease virus increased their protection against the Newcastle disease.

3.2 INTRODUCTION

Immunization of chickens with vaccines can prevent losses from clinical disease, including egg production declines and mortality. The design of vaccination programmes to accomplish protection for the vaccinated animals must be carefully done to minimize interference from maternal antibody (Ivanyi, 1970; Beard and Brugh, 1975; Westbury *et al*., 1984). The titration of serum for NDV hemagglutination inhibition (HI) antibody is a convenient way to evaluate the response to NDV vaccination (Beard and Brugh, 1975).

The degree of protection from morbidity or mortality is related to the virulence and dose of the challenge NDV, the route of infection, the age of the chicken at the time

of infection and the previous immunization history (Beard and Brugh, 1975). Most chickens with negative antibody titres (1:10) die if infected with virulent virus, while those with titres of 1:20 to 1:40 have a high probability of survival. Higher titres protected layers against egg production declines (Brugh, 1975; Allan *et al*., 1978; Westbury, 1984).

The ability of NDV and other paramyxoviruses to agglutinate red blood cells is due to the binding of the hemagglutin-neuraminidase protein to receptors on the surface of the red blood cell (Burnet, 1942). Chicken RBCs are usually used in hemagglutination (HA) tests, but Newcastle disease virus causes agglutination of all amphibian, reptilian and avian cells (Lancaster, 1975). Winslow *et al*. (1950), showed that human, mouse and guinea pig RBCs were agglutinated by all NDV strains tested but the ability to agglutinate cattle, goat, sheep, swine and horse cells varied with the strain of NDV. NDV and other paramyxoviruses may bring about hemolysis of RBCs or fusion of other cells by essentially the same mechanism (Ackerman, 1964). Attachment at the receptor site during replication is followed by fusion of the virus membrane with the cell membrane and this may result in the fusion of two or more cells. The rigid membrane of the RBCs usually results in lysis from the virus membrane fusion, leading to anemia in the NDV infected chicken (Ackerman, 1964).

Hyperproteinaemia can be caused by dehydration which is common in acute and chronic inflammation, liver disease, neoplasia, viral and rickettsial disease, fungal and protozoal disease (Bush, 1991). Plasma proteins increase with increase in age (Bush, 1991). Hypoproteinaemia results from inadequate protein consumption, small

intestine malabsoroption, liver disorder, congestive heart failure and increased protein loss through hemorrhage and burns (Bush, 1991).

Vaccination with live vaccine strains leads to increase in antibodies (Timm and Alexander, 1977). It has been shown that vaccinated chickens challenged with NDV can become infected due to stress, nutritional deficiencies, toxins, genetic immuno-deficiency, poor storage and administration of vaccines, rough handling of birds *et cetera* (Abdu *et al.,* 2006). Information on the effect of NDV challenge on vaccinated Shikabrown cocks is not available. This study was therefore designed to determine the effect of a velogenic Newcastle disease virus on the packed cell volume, total protein and hemagglutination inhibition antibody titres of vaccinated Shikabrown cocks.

3.3 MATERIALS AND METHODS

3.3.1 Location

The research was conducted at the Faculty of Veterinary Medicine, Ahmadu Bello University, Samaru, Zaria. Samaru is situated in the Northern Guinea savannah between latitudes 11º and 12ºN and between longitudes 7º and 8ºE at an elevation of 650 meters above sea level. Samaru has two seasons; dry (October to April) and rainy (May-October) with an annual rainfall of 1107 mm (Rekwot, 2000).

3.3.2 Experimental cocks

Fifty 20 week-old Shikabrown cocks consisting of 28 white and 22 red strains were purchased from the National Animal Production Research Institute Shika and used for this study. The cocks had been routinely vaccinated against ND, using the Vom produced vaccine, before purchase.

3.3.3 Management of cocks

The cocks were kept in twos inside cages and fed layers mash containing 18% crude protein, 95.6% dry matter, 17% crude fibre and 3% nitrogen *ad libitum*. Water was provided *ad libitum*. All the necessary screening and treatment for ecto, endo and hemoparasites were carried out. For a period of six weeks the cocks had their cloacal temperatures taken using a digital thermometer and were weighed weekly (Plate III). The cocks were infected with 2 ml of $10^{6.0}$ EID_{50} of a velogenic 113 strain Kudu of Newcastle disease virus intranasally and orally after screening.

3.3.4 Blood sampling

The fifty cocks consisting of 22 red Shikabrown and 28 white Shikabrown were sampled for 6 weeks post-infection. Twenty-five of the cocks consisting of 8 red Shikabrown and 17 white Shikabrown were infected with the virus. The cocks were bled weekly pre and post-infection for 6 weeks during which 2 ml of blood was taken from the wing vein for PCV and total protein determination. The blood was centrifuged at 251.6 g for 15 minutes to harvest sera for the determination of antibodies against Newcastle disease virus using hemagglutination inhibition test.

3.3.5 Determination of Newcastle disease antibody titres

***Hemagglutination test (testing for antigen titre)*:** Hemagglutination test was carried out by micro test method using two-fold serial dilution wells of 50 µl of reconstituted vaccinal virus antigen and 50 µl of 1% chicken red blood cells (Beard, 1980). An equivalent volume of chicken red blood cells suspension was also added to wells containing PBS with pH 7.2 alone to serve as control. The plate was gently tapped to

mix the contents and after 45 minutes of incubation at room temperature, the end point of the hemagglutination (HA) was read. The titre was taken as the reciprocal of the highest dilution giving a 100% agglutination of the 1% chicken RBCs. The last well that shows a complete hemagglutination is said to contain one hemagglutinating (HA) unit. If the seventh well (1:64) is the last to show complete hemagglutination, then the original material contained 64 (2^6) HA units. One HA unit is defined as the highest dilution of antigen which will completely agglutinate a test dose of red blood cells under standard conditions of temperature and time of incubation (Plate I).

Hemagglutination inhibition test (testing for semen antibody titre): The hemagglutination test method used is based on procedures described by Allan and Gough (1974). Beta technique (constant virus and varying serum) against Hemagglutination Agglutination Unit (HAU) of virus computed from the result of the HA titration was used. Double dilutions (50 µl) of the different chicken sera were reached with 50 µl of 4 HA units of the antigen suspension per well. The mixture was tapped gently to mix and allowed to stand for 30 minutes at room temperature for antigen-antibody reaction to take place. Antigen control wells were also included.

A 50 µl of 1% washed chicken red blood cells was added to all the wells and tapped to mix, incubated at room temperature and read after 45 minutes. The titres were taken as the reciprocal of serum dilutions giving 100% inhibition of the agglutination of red blood cells (Plate II).

3.3.6 Determination of packed cell volume

Hematocrit centrifuge technique was used to determine the packed cell volume (Schalm *et al*., 1975). Blood was collected from the wing vein of the cocks using heparinized capillary tubes on each day of collection once a week. The capillary tubes were placed on a centrifuge pad covered and centrifuged at 251.6 g for 15 minutes and PCV `was determined using the hematocrit reader.

3.3.7 Determination of total protein

After determination of the PCV the portion of the capillary tube containing the plasma was separated from the packed red cells. The plasma was then poured into the Goldberg refractometer or Total Solids (TS) meter and the reading taken.

3.3.8 Statistical analysis

Data on PCV, TP and HIT were subjected to analysis of variance using the statistical analysis system (SAS) and differences between treatment means were determined by Duncan's multiple range tests (Helwig and Council, 1979).

3.4 RESULTS

None of the cocks died as a result of the challenge with the velogenic NDV. The packed cell volume of the control red cocks (RSB_1)(41.9±0.9) was slightly higher than that of the control white cocks (41.2±1.0) and infected red (41.1±1.2) and white cocks (39.5±0.8) at week 1 post infection. The infected red and control white cocks had almost the same packed cell volume value at week 1 (WSB_1; 41.2±1.0; RSB_2; 41.1±1.2). At week 2 the PCV of the control and infected red cocks were almost the same (Table 3.1; Fig. 3.1). The control white cocks had slightly lower packed cell

volume (WSB_1, 34.5±0.8) than the infected white cocks (WSB_2; 38.5±0.7). Both the control and infected red cocks had higher PCV than the control and infected white cocks at week 2 (Table 3.1; Fig. 3.1).

The packed cell volume of the control white cocks at week 3 was higher than that of the control red cocks and infected red and white cocks (Table 3.1; Fig. 3.1). The PCV of the control and infected red cocks was about the same at week 3. At week 4 the PCV of the control white cocks was higher than that of the control red cocks and infected red and white cocks. The PCVs of the control red cocks and infected red and white cocks were almost of the same value (Table 3.1; Fig. 3.1).

The PCV of the control white cocks was again higher than that of the conrol red cocks and infected red white cocks at week 5 (Table 3.1; Fig. 3.1). The infected red cocks had the least PCV followed by the infected white cocks (Table 3.1; Fig. 3.1). The PCVs of the control and infected red cocks were almost the same at week 6. The PCV of the control white cocks was the lowest (Table 3.1). There was no significant difference in the PCV of the control and infected red cocks, the infected cocks had slightly lower PCV (Table 3.1; Fig. 3.1).

The total protein values of the control and infected red cocks were about the same at week 1, 2, 3, 4, 5 and 6 (Table 3.1; Fig. 3.2). The total protein of the infected white cocks was slightly lower than that of the control cocks at week 1, 2, 3 and slightly higher at week 4, 5 and 6 (Table 3.1; Fig. 3.2).

The control red cocks had significantly ($P<0.05$) lower antibody titres than the infected red cocks at week 1, 2, 3, 4, 5 and 6. Similarly, the control white cocks had significantly ($P<0.05$) lower antibody titres than the infected white cocks throughout the six week period of the research work (Table 3.1; Fig. 3.3).

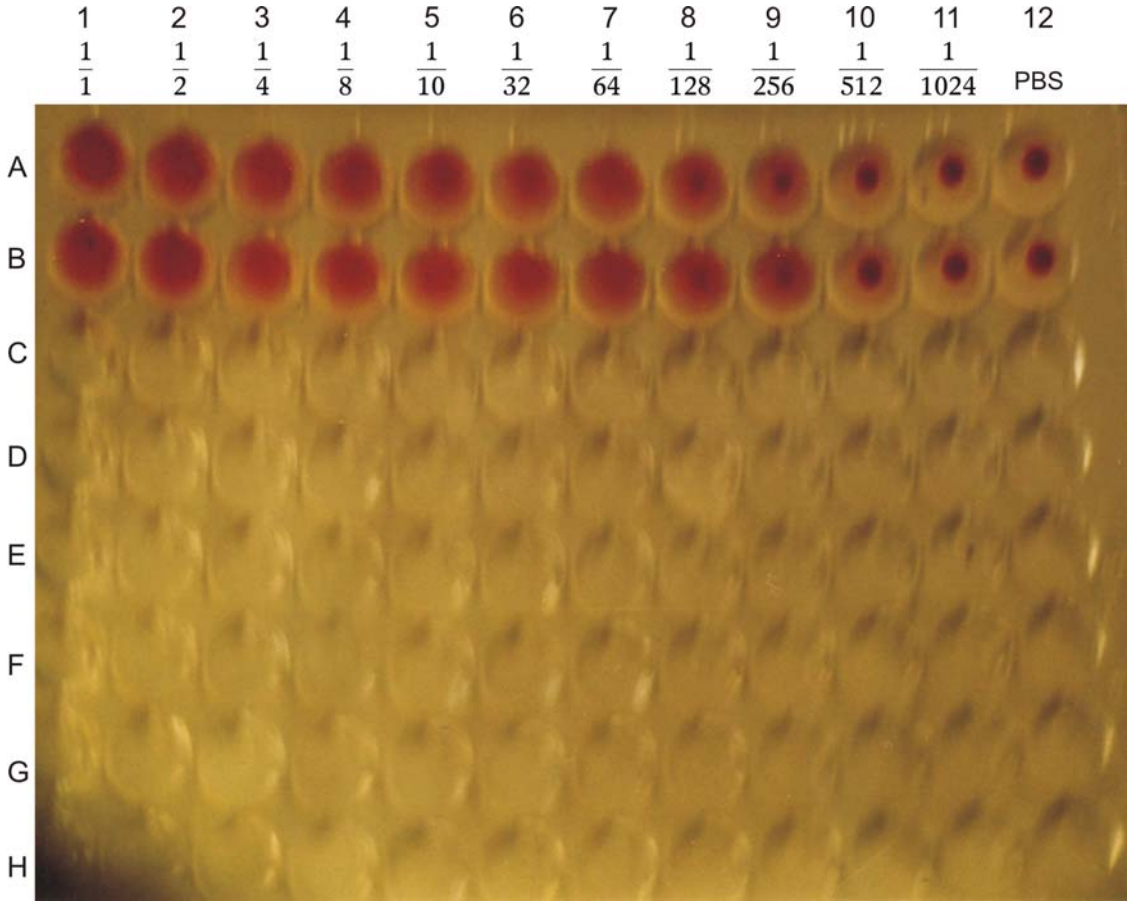

Plate I. Hemagglutination test (HA Test)(testing for the presence of ND virus)

Plate I shows a thin film of red blood cells indicating the presence of hemagglutinin. Wells No. 1 - 7 show a complete hemagglutination. Well 8 – 12 show a sharp button of red blood cells indicating the absence of hemagglutination. The sharp buttons of red blood cells are not very sharp in wells No. 8 and 9 but can still be seen.

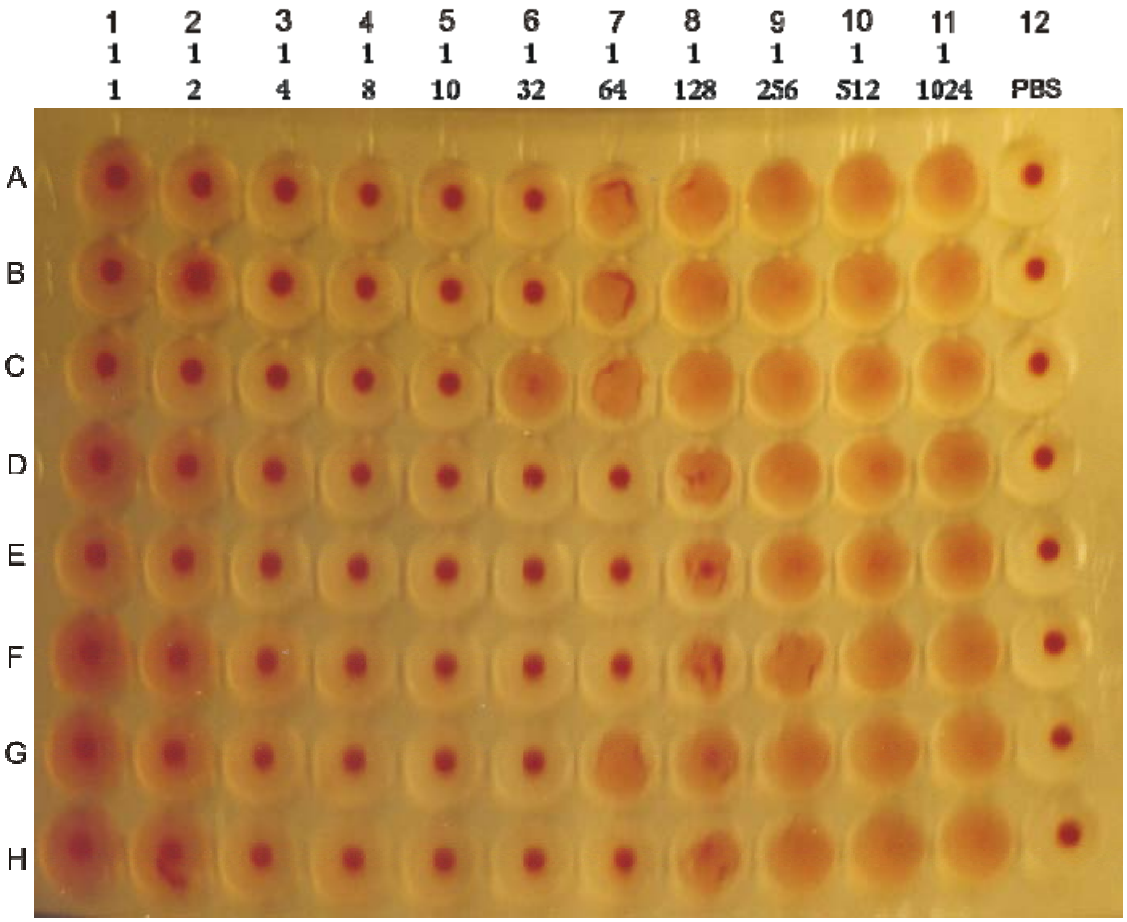

Plate II. Hemagglutination inhibition test (HIT)(testing for ND antibodies)

The wells with sharp buttons of red blood cells show hemagglutination inhibition while the wells with a thin film of red blood cells show lack of hemagglutination inhibition.

Table 3.1: Packed cell volume (PCV), hemagglutination inhibition antibody titres (HIT) and total protein of control and infected Shikabrown cocks

		Week 1		Week 2		Week 3		Week 4		Week 5		Week 6	
Parameter	Breed	Control	Infected	Control	Infected	Control	Infected	Control	Infected	Control	Infected	Control	Infected
Hemagglutination	RSB	4.5±0.5a	7.0±0.7b	4.0±0.5a	9.4±0.6b	3.1±0.4a	8.5±0.5b	2.9±0.4a	8.13±0.5b	2.93±0.4a	7.0±0.5b	1.5±0.3a	5.4±0.4b
inhibition antibody titres (HIT)	WSB	3.4±0.6	8.0±0.5	4.0±0.5	9.4±0.4	2.4±0.4	7.8±0.3	2.18±0.4	8.4±0.4	2.64±0.4	7.4±0.4	1.1±0.3	5.47±0.3
Packed cell	RSB	41.9±0.91	41.1±1.2	39.6±0.7	39.03±0.1	41.7±1.3	41.5±1.7	41.5±0.1	41.1±1.3	43.9±1.1	40.6±1.5	43.5±0.9	43.13±1.2
volume (PCV)	WSB	41.2±1.0	39.5±0.8	34.5±0.8	38.5±0.7	46.1±1.5	39.9±1.2	46.3±1.1	41.0±0.9	46.0±1.2	42.0±1.0	39.2±1.0	40.3±0.8
Total protein (TP)	RSB	4.6±1.6	4.5±2.0	4.5±0.1	4.5±0.2	4.75±0.1	4.9±0.2	4.5±0.1	4.8±0.2	4.6±0.2	4.5±0.4	4.7±0.1	4.69±0.2
	WSB	4.7±1.8	4.1±1.4	4.6±0.1	4.39±0.1	4.9±0.1	4.6±0.1	4.6±0.2	4.7±0.1	4.65±0.2	5.0±0.1	4.76±0.2	4.9±0.1

ab = Means with different letter superscripts within treatments are significantly (P<0.05) different within rows.

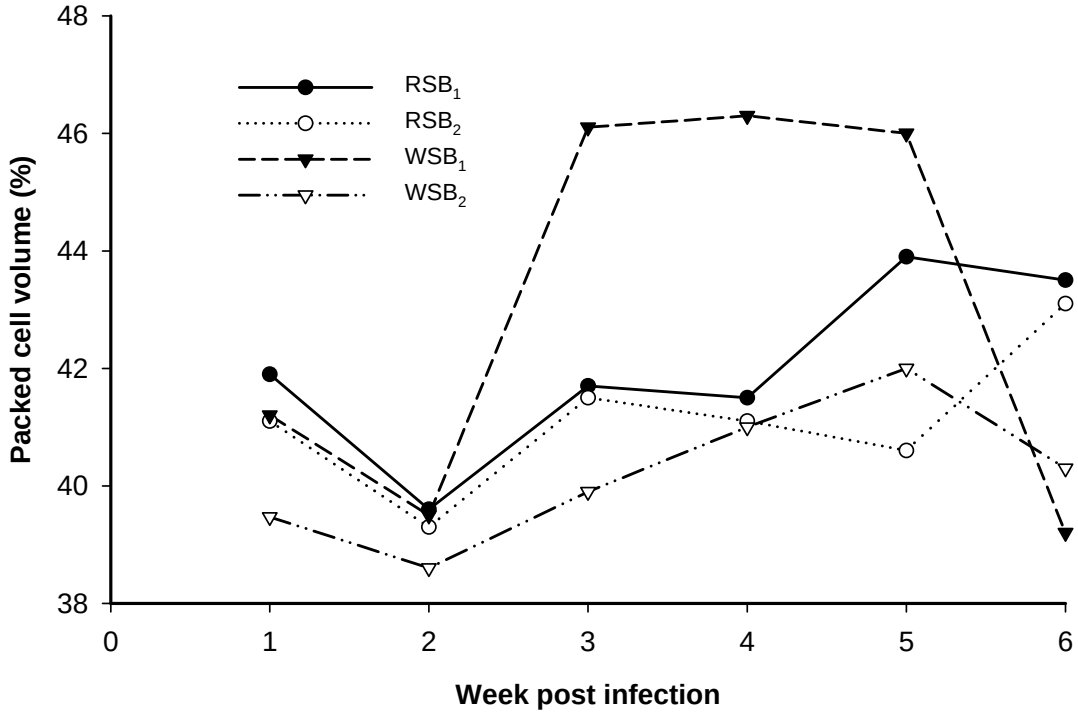

Fig. 3.1 Packed cell volume for control and infected Shikabrown cocks

Note:

RSB_1 = Red Shikabrown control
RSB_2 = Red Shikabrown infected
WSB_1 = White Shikabrown control
WSB_2 = White Shikabrown infected

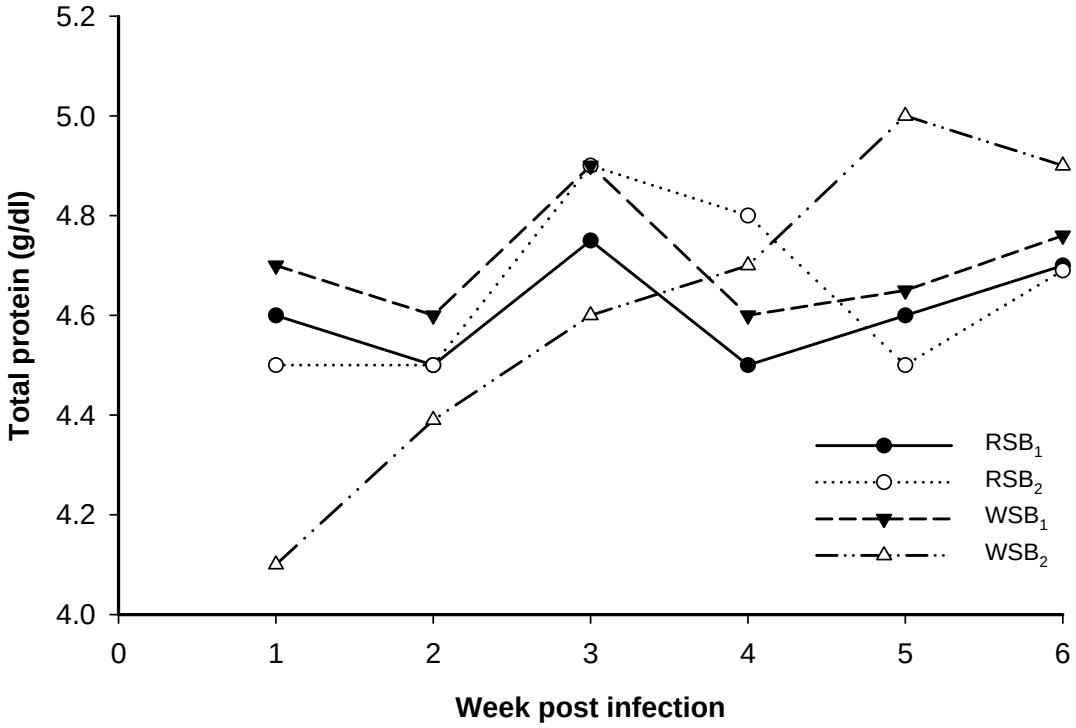

Fig. 3.2 Total protein of control and infected Shikabrown cocks

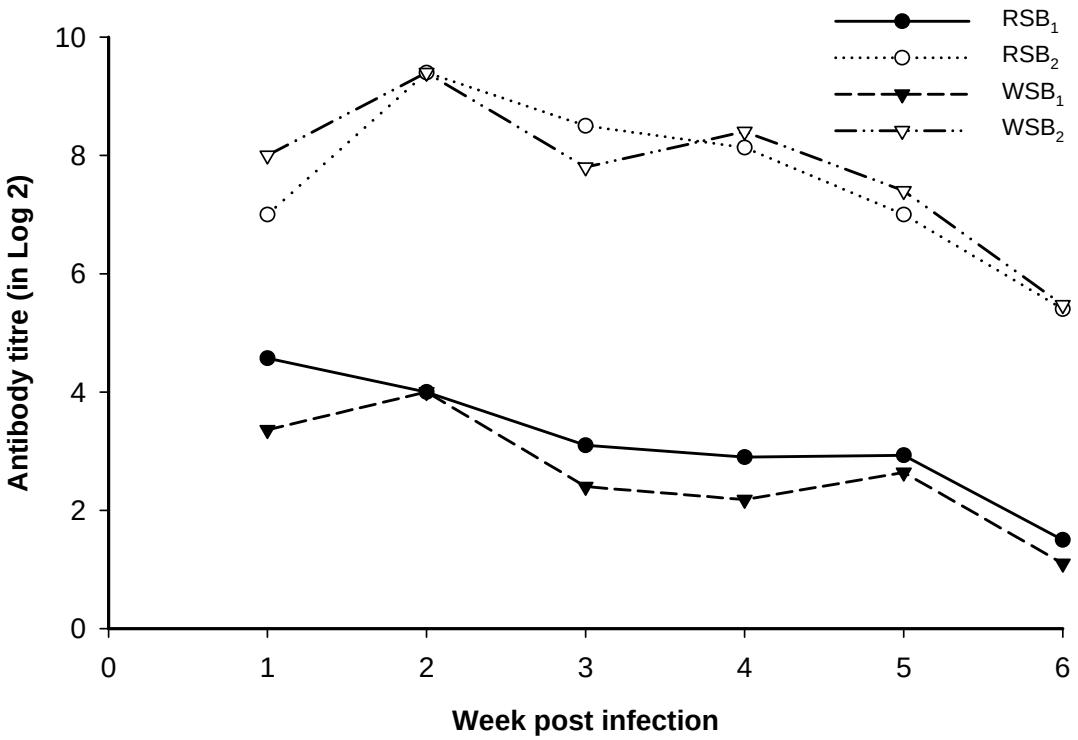

Fig. 3.3 Hemagglutination inhibition Newcastle disease antibody titre of control and infected Shikabrown cocks

3.5 DISCUSSION

In this study there was no significant decrease in the mean packed cell volume post infection throughout the six week period of observation in both the red and white cocks. This disagreed with the findings of Oladele (2004) who found a decrease in the packed cell volume 2 days post infection and which continued up to day 11. The cocks used in this study were vaccinated hence had immunity against Newcastle disease virus which prevented destruction of their red blood cells by the velogenic Newcastle disease virus.

There was no significant difference ($P>0.05$) between the mean total protein values of the control and infected red and white cocks used in this study. Oladele (2004), however found a decrease in the total protein values from day 3 and it reached its lowest value of 2.6±0.15 g/dl by day seven post infection. This was followed by a gradual increase until day 21 when there was a slight drop in value and stabilized by day 42 post infection. His finding disagreed with the present finding probably due to the differences in breed of chickens used and the vaccination history of the cocks used by Oladele (2004) and in the present work. In this study vaccinated Shikabrown cocks at 20 weeks old were used while Oladele used day old Shaver brown unvaccinated chicks.

The high mean antibody titres of the infected red and white cocks showed that challenging the cocks with the velogenic virus increased the antibody titres of the cocks giving them more protection from infection by the Newcastle disease virus. The highest mean antibody titre in this work was 9.4±0.6 $\log_2$ for infected cocks at 2

weeks post infection and thereafter decreased to the lowest level of 5.4±0.4 log_2 at week 6 post infection. This agrees with the findings of Oladele (2004) who infected Brown Shaver birds with Kudu strain 113 NDV and reported that antibody titres increased by day 2 post infection up to a maximum value of 9.7±1.2 log_2 by day four post infection and thereafter decreased.

The mean antibody titre of 1.9±0.7 to 4.6±0.4 log_2 provided protection to the red and white cocks against the velogenic Newcastle disease virus. In an endemic environment like Zaria farmers keeping red and white Shikabrown chickens should vaccinate them against Newcastle disease. The red Shikabrown cocks with slightly higher mean antibody titres withstood the challenge by the velogenic Newcastle disease virus better than white Shikabrown cocks. The packed cell volume of the red Shikabrown cocks was slightly higher than that of the white Shikabrown cocks because they had higher mean antibody titres and withstood the challenge of the Newcastle disease virus better than the white Shikabrown cocks.

The red Shikabrown cocks reacted better to vaccination than the white Shikabrown cocks. The slightly higher antibody titre in the red Shikabrown cocks is an indication that they responded better to previous vaccination against Newcastle disease; based on this finding it was expected that the red Shikabrown cocks will better resist infection by the Newcastle disease virus than the white Shikabrown cocks. This speculation has been strengthened by the fact that the changes in the mean packed cell volume of the red Shikabrown cocks was not affected as much as that of the white Shikabrown cocks after challenge with the velogenic Newcastle disease virus.

From this study hemagglutination inhibition Newcastle disease antibody titres of as low as 2 $\log_2$ were able to protect birds challenged with the velogenic Newcastle disease virus used. This value was below the recommended value of 5 $\log_2$ (Allan *et al.*, 1978). It is therefore possible that birds with antibody titres of less than 5 $\log_2$ will be protected when exposed to a velogenic Newcastle disease virus.

The study also confirmed that vaccinated birds could be infected with velogenic Newcastle disease virus when exposed. The challenge of the red Shikabrown and white cocks with the velogenic Kudu 113 strain of Newcastle disease virus in this study led to an increase in antibody titres in the cocks hence more protection from Newcastle disease infection.

CHAPTER 4

SEMEN CHARACTERISTICS OF VACCINATED SHIKABROWN COCKS CHALLENGED WITH A VELOGENIC NEWCASTLE DISEASE VIRUS

4.1 ABSTRACT

Fifty 20-weeks old Shikabrown cocks consisting of 22 red Shikabrown cocks and 28 white Shikabrown cocks were purchased from the National Animal Production Research Institute Shika. The cocks were fed on a diet of layers mash with 18% crude protein, 95.6% dry matter, 17.1% crude fibre and 3% nitrogen.

Twenty-five cocks consisting of 8 red and 17 white Shikabrown cocks selected on the basis of body weight and antibody titres were infected with 0.2 ml of $10^{6.0}$ EID_{50} of a velogenic Kudu 113 strain of Newcastle disease virus intranasaly and orally. Twenty-five cocks consisting of 14 red and 11 white Shikabrown cocks served as controls. Cloacal temperatures, body weights and semen samples of both control and infected cocks were taken weekly for six weeks. The semen was evaluated for volume, colour, motility, concentration, per cent live spermatozoa and percentage total spermatozoa abnormalities. Semen colour was graded as creamy (1 = very good); milky (2 = good) and watery (3 = poor).

There was no significant difference in the cloacal temperatures and body weights of control and infected red and white cocks. The infected red and white cocks had slightly higher cloacal temperatures and body weights.

The semen volume of infected red cocks showed a general increase over that of control red cocks. The semen volume of the control white Shikabrown cocks was significantly higher than that of the infected white cocks. The white Shikabrown cocks had higher semen volume than the red Shikabrown cocks. The red Shikabrown cocks had slightly better semen colour than the white Shikabrown cocks. The control white cocks had better spermatozoa motility than the infected white cocks, while the infected red cocks had better spermatozoa motility than the control red cocks. The white Shikabrown cocks generally had better spermatozoa motility than the red cocks.

The spermatozoa concentration of the control white cocks was consistently better than that of the infected white cocks, the reverse was the case with the red cocks where the spermatozoa concentration of the infected red cocks was higher than that of the control red cocks. The white cocks had better spermatozoa concentration than the red cocks.

The control white Shikabrown cocks had significantly ($P<0.05$) higher per cent live spermatozoa than the infected white cocks. The infected red Shikabrown cocks had significantly ($P<0.05$) higher per cent live spermatozoa than the control red cocks. The control white cocks had significantly ($P<0.05$) higher per cent live spermatozoa than the control red cocks. The infected red and white Shikabrown cocks had higher percentage total spermatozoa abnormalities than the control red and white cocks.

It can be concluded from this study that the white Shikabrown cocks had better semen quality than the red Shikabrown cocks. In addition the Newcastle disease virus in non-infected cocks had better semen quality than the infected.

It is recommended that white Shikabrown cocks should be used for breeding purposes. It is also recommended that breeder cocks should be routinely vaccinated against Newcastle disease to ensure that the level of antibodies is high enough to prevent adverse effect on semen quality. To ascertain adequately whether the abnormalities observed in infected cocks can be translated into infertility in breeder hens further breeding studies need to be conducted.

4.2 INTRODUCTION

There have been several studies conducted on semen production and quality in a variety of breeds of poultry. Semen production is strongly correlated with testis size, body weight and breed (Burrows and Titus, 1939; Williams and McGibbon, 1943; Boone and Hughes, 1969). Seasonal variations in semen production have also been reported in poultry (Wheeler and Andrews, 1943; Polge, 1951; Kamar and Nadreldin, 1959; Saeid and Al-Soudi, 1975). Reproductive inefficiency is recognized as the costliest and limiting constraint to efficient animal production. It is also well known that both qualitative and quantitative characteristics of semen have a marked effect on egg fertility (Kamar, 1960; Chalow, 1970). Gbadamosi and Egbunike (1999) have shown that the frequency of ejaculation could have adverse effect on the quality of the ejaculates and the fertilizing capability of the sperm cells.

In cocks, the paired testes are attached to the dorsal wall of the abdomen on either side of the midline adjacent to the anterior parts of the kidneys and just posterior to the lungs (Lake and Stewart, 1978; Hafez, 1987). Semen is produced in the testes and stored in the ductus deferens, which extends tortuously from the testes and ends in

two papillae or wart-like projections (phallus) into the cloacae (Lake and Stewart, 1978). Little attention has been devoted to the male's role in improving egg fertility of domestic fowl with the exception of progeny testing and selection of males on the basis of sib performance (Jones and Lemoreux, 1942; Marks, 1981). It is imperative that male animals should be in good physical and sexual health for natural mating or collection of good quality semen for artificial insemination (Zemjanis, 1970; Roberts, 1971).

Decreased egg production was experimentally produced by infecting breeder turkey hens with Eastern Equine Encephalitis virus (EEEV) or Highland J. virus (Guy *et al*., 1995). The viruses were shed in the semen of turkey toms infected experimentally (Guy *et al*., 1995). This proved that these viruses can be transmitted sexually through artificial insemination of turkey hens and using infected semen. This spreads the viruses leading to decreased egg production. It was also observed that a decline in egg production due to Western Equine Encephalitis virus (WEEV) was accompanied by an increase in semen antibody titres to WEEV between acute and convalescent sera in turkeys from affected flocks (Cooper and Medina, 1997).

Factors affecting reproduction in male animals include age, breed, nutrition, season and disease. The semen characteristic of productive males are relatively unaffected by the dietary intake of protein (Hockings, 1989). Males consuming low amounts of protein ejaculate more frequently and their lifetime sperm output far exceeds that of males consuming high amount of protein (Hockings, 1989; Duncan *et al*., 1990; Etches, 1996). The proportion of males that ejaculate in response to abdominal massage is maximized when protein intake is about 10 g/day (Hockings, 1989). In

males, unrestricted access to feed is associated with fat cocks which develop leg and joint deformities and atrophied testes (Hockings, 1989). The combination of joint weakness and fatness of the cock reduces the success and not the frequency of mating and consequently the fertility of flocks in which the males consume excess feed is poor, particularly after 50 weeks of age (Hockings, 1989).

Nutritional deficiencies may impair reproduction by producing their primary effect on a variety of tissues and organs, resulting in non-specific decreased reproductive performance (Gerioff, 1986). Such nutrient imbalances may primarily affect the anterior pituitary or hypothalamus, thus interfering with normal luteinizing hormone and follicle stimulating hormone production (Gerioff, 1986).

Apart from the general debilitating effects of disease on the health of an animal, reproductive diseases impair reproductive activities and thus reduce productivity in animals (Rekwot *et al*., 1998). Viral and bacterial diseases such as Newcastle disease, salmonellosis, egg drop syndrome and fowl typhoid affect egg production in poultry (Jordan, 1990).

Newcastle disease is widespread in Nigeria and enzootic in Zaria. Newcastle disease is known to cause decrease in egg production. It is uncertain if there is any record up till now on the effect of ND on semen characteristics. This study was therefore designed to determine the effect of a velogenic Newcastle disease virus on the semen characteristics of vaccinated Shikabrown cocks.

4.3 MATERIALS AND METHODS

4.3.1 *Location*

The location of this study is as described in 3.3.1.

4.3.2 *Experimental cocks and management of cocks*

Experimental cocks and management of cocks are as described in 3.3.2 and 3.3.3.

4.3.3 Infection of cocks with velogenic Kudu 113 strain of Newcastle disease virus

Twenty-five cocks consisting of 8 red Shikabrown and 17 white Shikabrown were infected with a velogenic Kudu 113 strain of Newcastle disease virus. The virus was described by Echeonwu *et al*., 1993, and had the following characteristics; hemagglutination titre of ($\log_2$ 256); mean lethal dose of (log 108.00); mean death time of (49.60 h); intracerebral pathogenecity index of (1.56); intravenous pathogenecity index dose of 2.18; embryo infective dose, 50 per cent endpoint per ml 8.46; per cent adsorption of chicken brain cell (97.66%); thermostability of hemagglutination at 56ºC (120 minutes) and virus elution rate (>26 h). The titre of the NDV was $10^{8.5}$ ml^{-1} (Alexander, 1988). A vial of the NDV was dissolved in 63 ml of phosphate buffered saline (pH 7.4) and each cock was inoculated with 0.2 ml intranasaly and intraocularly.

4.3.4 *Semen collection*

Semen was collected according to the method described by Lake and Stewart (1978). This involved a gentle massage (stroking) of the back feathers two or three times with the palm of the hand and the abdomen towards the tail with the other hand simultaneously.

The semen was immediately expressed from the swollen ejaculating papillae into a graduated plastic tube (Plates IV and V).

4.3.5 *Semen evaluation*

Immediately after collection, all semen samples were immersed in a flask containing water at 42°C and carried to the laboratory for immediate evaluation. The semen samples were evaluated for volume, color, motility, concentration, per cent live spermatozoa and percentage total sperm abnormalities as described by Zemjanis (1970). This included (i) visual and gross evaluation of the ejaculate soon after collection with respect to volume, colour and presence or absence of foreign materials and (ii) microscopic examination of wave pattern (gross motility) and live-dead counts. The semen colour was graded as creamy (1 = very good), milky (2 = good) or watery (3 = poor). Gross motility was determined by examining a drop of raw and undiluted semen on a pre-warmed slide under a light microscope at 100 x magnification. The concentration of the spermatozoa was determined using the red blood cell counting chamber of a hemocytometer that was crossed with microscopic grids containing 25 large squares with each containing smaller squares. Sperm cells were counted diagonally from the left top to the right bottom in five large squares or a total of 80 small squares (Coles, 1980). Semen smears were stained with eosin-nigrosin for the determination of live-dead ratio. Live sperm cells repel the stain and were colorless, while dead cells absorb the stain and appeared reddish (Coles, 1980). At least 400 spermatozoa per slide were counted using the phase contrast microscope at x 40 magnification with oil immersion. Fresh raw semen samples were also fixed in buffered formol saline for two to three days to determine sperm abnormalities. About 400 spermatozoa per slide were counted using the phase contrast microscope at 40 magnification with oil immersion (Coles, 1980).

Plate III. Taking of cloacal temperature using a digital thermometer.

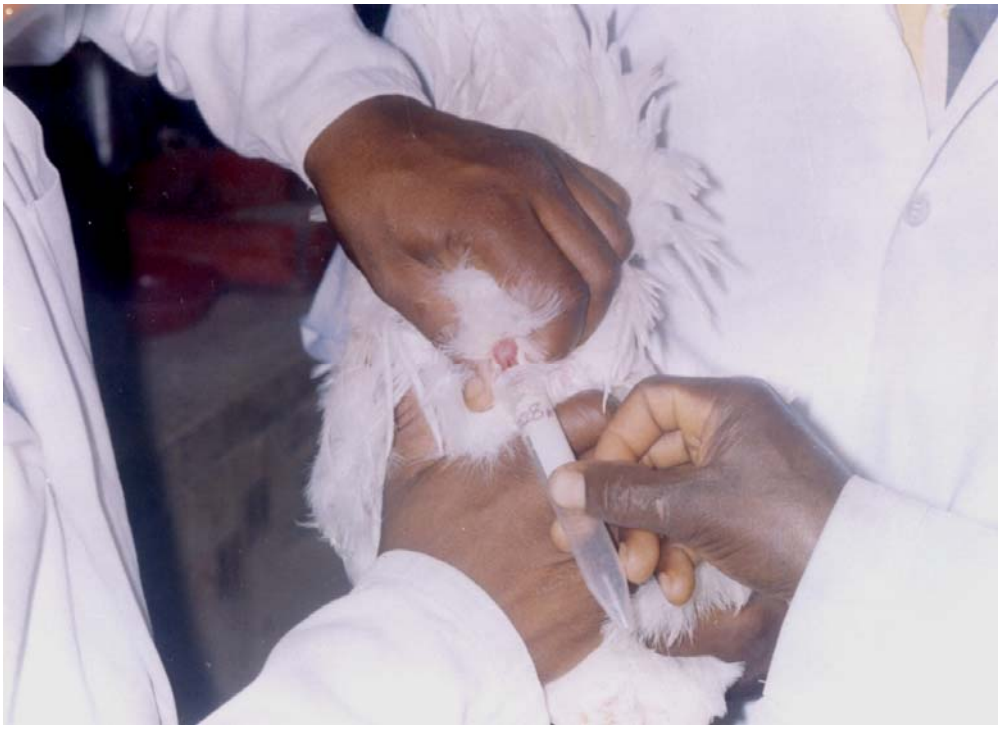

Plate IV. Semen collection using a graduated plastic tube

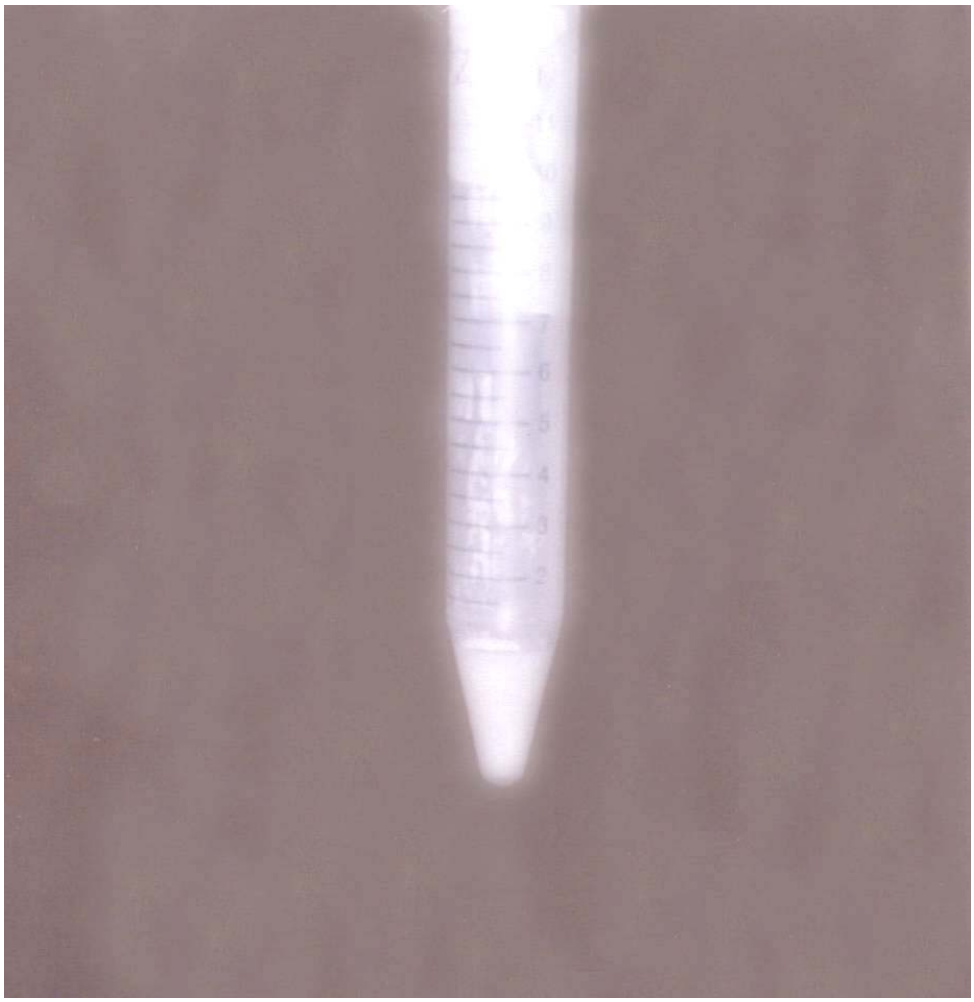

Plate V. Semen sample in a graduated plastic tube

4.3.6 *Statistical analysis*

Data of the cloacal temperatures, body weights and semen parameters were analysed using the analysis of variance procedure and differences between treatment means compared by Duncan's Multiple Range Tests (Helwig and Council, 1979).

4.4 RESULTS

4.4.1 *Cloacal temperatures and body weight*

There was no significant difference in the cloacal temperatures of control and infected red Shikabrown cocks. Similarly there was no significant difference in the cloacal temperatures of the control and infected white Shikabrown cocks. The infected red and white cocks had slightly higher cloacal temperatures (Fig. 4.1).

The body weights of the control and infected red Shikabrown cocks were not significantly different, the same was also true of the control and infected white Shikabrown cocks. The infected red and white cocks were slightly lighter in weight than the control cocks (Fig. 4.2).

4.4.2 *Semen volume and colour*

The semen volume of the control and infected red Shikabrown cocks was not significantly different at week 1, 2, 3, 4, 5 and 6. The semen volume of the infected red cocks showed a general decrease over that of the control red cocks. The semen volume of the control white cocks was significantly higher than that of the infected white cocks at week 1 and 2. There was no significant difference between the semen volume of the control and infected white cocks at week 3, 4, 5 and 6. The semen volume of the infected white cocks showed a general decrease (Fig. 4.3). The white Shikabrown cocks had higher semen volume than the red cocks.

The semen colour of the control and infected red cocks and control and infected white cocks did not show any significant difference (Fig. 4.4).

4.4.3 *Spermatozoa motility*

Spermatozoa motility of the control and infected red Shikabrown cocks was not significantly different at week 1 and 6 post infection. The motility of the control red cocks was significantly higher ($P<0.05$) than that of the infected red cocks at week 2, 3, 4 and 5 the control red cocks had a significantly ($P<0.05$) higher spermatozoa motility than the infected red cocks (Fig. 4.5).

The control white Shikabrown cocks had significantly higher spermatozoa motility than the infected white cocks at week 1, 2, 3, 4, 5 and 6. The infected red cocks had lower spermatozoa motility than the control red cocks while the control white cocks had significantly higher spermatozoa motility than the infected white cocks. The white Shikabrown cocks generally had better spermatozoa motility than the red Shikabrown cocks (Fig. 4.5).

4.4.4 *Spermatozoa concentration*

The spermatozoa concentration of the control red Shikabrown cocks was significantly higher at week 3, 4 and 5 (Fig. 4.6). The infected red cocks had significantly lower spermatozoa concentration than the control red cocks (Fig. 4.6). The spermatozoa concentration of the control white Shikabrown cocks was significantly higher than that of the infected white cocks at week 1, 2 and 3. There was no significant difference ($P>0.05$) in the spermatozoa concentration of the control and infected white cocks at week 4, 5 and 6. The spermatozoa concentration of the control red and white cocks was persistently higher

than that of the infected red and white cocks throughout the period of the research. The white cocks had generally better spermatozoa concentration than the red cocks (Fig. 4.6).

4.4.5 *Per cent live spermatozoa*

The control red Shikabrown cocks had significantly higher per cent live spermatozoa than the infected red cocks at week 1, 2, 3, 4 and 6 post infection (Fig. 4.7). At week 5 there was no significant difference between the infected and control cocks ($P>0.05$).

The control white Shikabrown cocks had significantly higher percentage live spermatozoa ($P<0.05$) than the infected white cocks at week 1, 2, 3, 4, 5 and 6 (Fig. 4.7). The per cent live spermatozoa of the control white Shikabrown cocks was significantly higher ($P<0.05$) than that of the control red Shikabrown cocks (Fig. 4.7).

4.4.6 Percentage total spermatozoa abnormalities

There was no significant difference ($P>0.05$) in the percentage total spermatozoa abnormalities of the control and infected red cocks at week 1 and 3 (Fig. 4.8). The infected red cocks had a significantly higher percentage total abnormalities at week 2, 4, 5 and 6 (Fig. 4.8). The infected white Shikabrown cocks had significantly higher percentage total abnormalities than the control at week 1, 2, 5 and 6. There was no significant difference ($P>0.05$) in the percentage total abnormalities between the control and infected white cocks at week 3 and 4.

Table 4.1: Effect of Newcastle disease virus on body weight, cloacal temperature and semen characteristics of red Shikabrown and white cocks

		Week 1		Week 2		Week 3		Week 4		Week 5		Week 6	
Parameters	Breed	Control	Infected	Control	Infected	Control	Infected	Control	Infected	Control	Infected	Control	Infected
Cloacal	RSB	41.7±0.2	41.8±0.68	41.6±0.1^{a}	41.8±0.1^{b}	41.6±0.9	41.13±0.4	41.5±0.3	41.8±0.1	41.7±0.1	42.1±0.1	41.4±0.1	41.7±0.9
temperature (ºC)	WSB	41.0±0.3	41.8±0.78	41.4±0.1^{b}	41.7±0.1^{b}	41.5±0.7	41.7±0.7	41.5±0.6	42.3±0.1	41.6±0.1	41.9±0.1	41.66±0.0	41.5±0.9
Body weight (kg)	RSB	2.2±0.2^{a}	2.1±0.2^{b}	2.2±0.1	2.1±0.2	2.3±0.1^{a}	2.1±0.1^{b}	2.2±0.1	2.1±0.1	2.2±0.0^{a}	2.2±0.1^{a}	2.2±0.1^{a}	2.2±0.1^{b}
	WSB	2.2±0.2^{a}	2.2±0.17^{b}	2.2±0.1	2.2±0.0	2.1±0.07^{a}	2.0±0.1^{b}	2.2±0.1	2.2±0.1	2.3±0.1^{a}	2.2±1.0^{b}	2.3±0.1^{a}	2.2±0.4^{b}
Semen volume (ml)	RSB	0.3±0.1	0.3±0.08	0.3±0.3	0.2±0.4	0.3±0.1	0.3±0.1	0.3±0.1	0.2±0.1	0.3±0.3	0.3±0.1	0.3±0.1	0.3±0.1
	WSB	0.3±0.1	0.2±0.05	0.8±0.3	0.5±0.3	0.5±0.1	0.5±0.1	0.4±0.1	0.3±0.1	0.4±0.1	0.3±0.1	0.5±01	0.5±0.1
Color (1-3)	RSB	1.6±0.4	1.3±0.3	1.6±0.4	1.4±0.3	1.9±0.2	1.3±0.2	2.0±0.4	1.6±0.3	2.1±0.3	1.3±0.4	1.8±0.3	1.6±0.4
	WSB	2.1±0.3	1.2±0.27	1.9±0.3	2.0±0.3	2.4±0.3	1.7±0.2	1.9±0.3	2.5±0.3	2.1±0.3	1.9±0.3	2.8±0.4	2.4±0.3
Sperm motility (%)	RSB	38.0±11.4	35.7±8.6	53.1±0.9	41.8±6.8	73.8±0.8	42.9±0.6	60.6±9.8	47.1±7.5	55.7±7.7	32.5±0.1	50.7±0.8	50.0±0.1
	WSB	70.5±9.7	43.8±0.7	73.6±7.7	55.6±6.2	66.8±0.7	60.3±5.7	61.2±6.8	55.9±8.4	60.0±8.7	55.0±0.7	69.5±9.8	57.2±7.5
Sperm	RSB	2.3±0.6	1.7±0.5	3.2±0.8	2.3±0.6	4.1±0.7	1.9±0.5	3.6±1.5	2.5±1.2	3.8±0.3	1.5±0.5	2.6±0.6	2.4±0.8
concentration (x10^{9}/ml)	WSB	2.7±0.6	1.8±0.5	5.0±0.7	3.6±0.5	3.8±0.6	2.6±0.5	4.9±1.3	4.1±1.1	3.6±0.3	2.8±0.7	5.0±0.7	4.6±0.6
Per cent live	RSB	38.2±0.9^{a}	38.2+0.9^{a}	64.4±0.1^{a}	53.9±0.1^{b}	69.4±0.9^{a}	40.0±0.7^{b}	63.1±0.1^{a}	58.2±0.8^{b}	56.8±0.8	54.9±0.1	54.7±0.1^{a}	47.5±0.1^{b}
spermatozoa (%)	WSB	69.5±0.1^{a}	45.0±0.8^{a}	75.9±0.9^{a}	66.2±0.7^{b}	76.8±0.8^{a}	66.2±0.6^{b}	63.8±0.7^{a}	57.7±0.9^{b}	72.3±0.9^{a}	40.1±0.7^{b}	80.0±0.1^{a}	66.2±0.8^{b}
Total	RSB	3.1±1.3	3.3±0.1	3.6±0.2^{a}	7.4±2.6^{b}	6.4±1.3	6.6±1.7	3.6±4.8^{a}	6.2±3.6^{b}	2.4±0.7^{a}	7.0±0.9^{b}	2.7±0.9^{a}	5.1±1.4^{b}
abnormalities (%)	WSB	3.9±0.9	6.4±1.2	3.9±2.2	9.8±1.8	5.8±1.4	6.1±1.2	5.0±4.1	6.3±3.3	3.0±0.8	7.6±07	3.8±1.2	6.6±0.9

ab = Mean with different letter superscripts within treatments and breeds are significantly (P<0.05) different within rows.

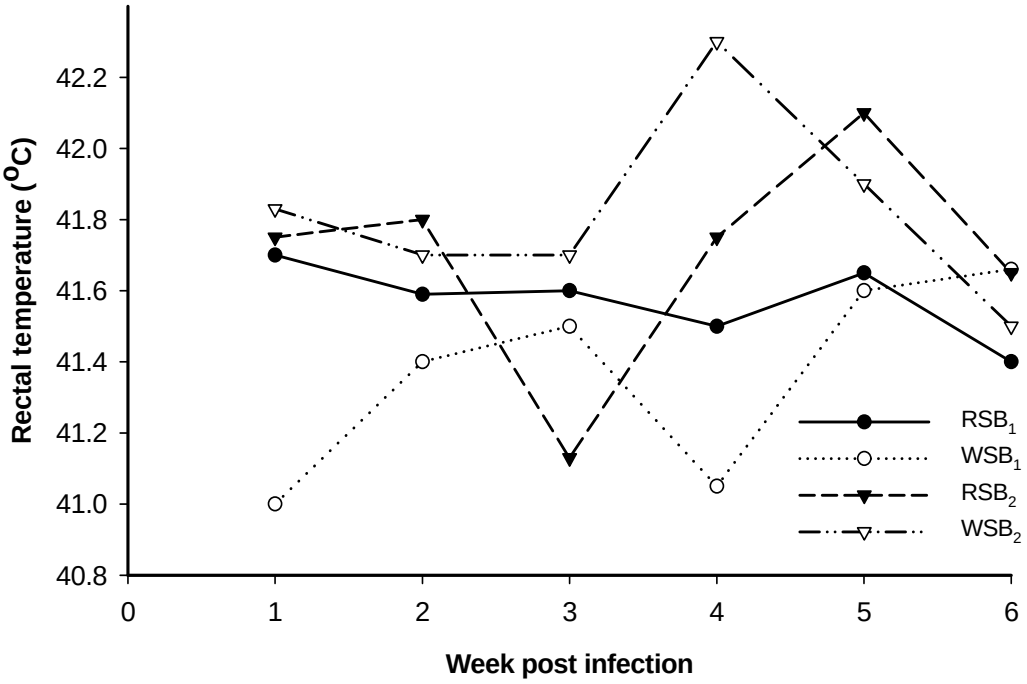

Fig. 4.1 Rectal temperature of control and infected Shikabrown cocks

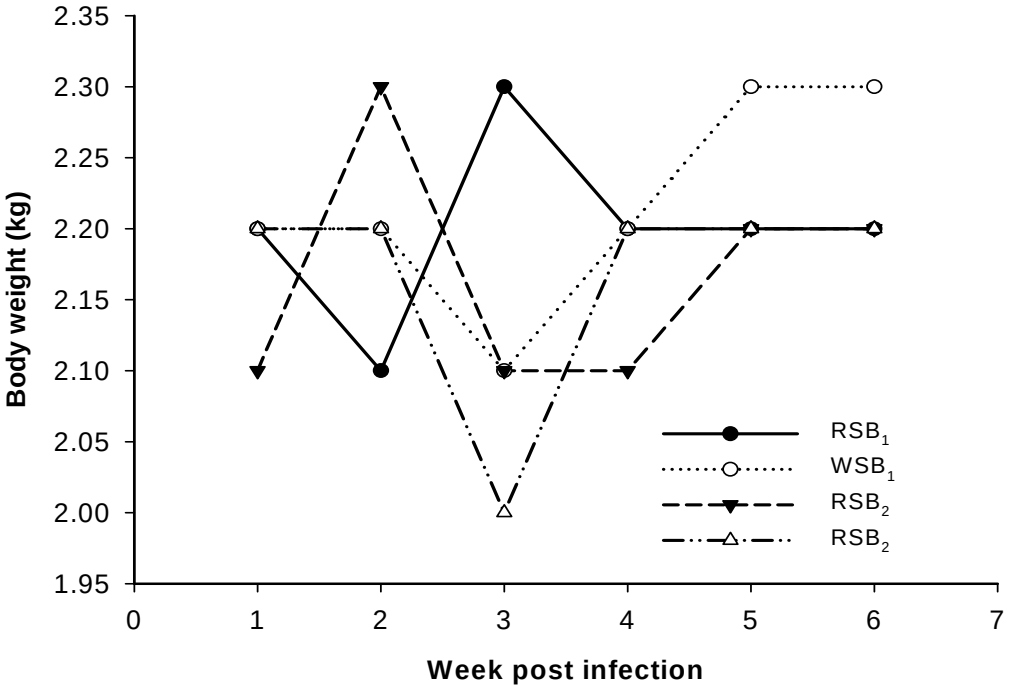

Fig. 4.2 Body weight of control and infected Shikabrown cocks

Key

RSB_1 = Control red Shikabrown cocks
WSB_1 = Control white Shikabrown cocks
RSB_2 = Infected red Shikabrown cocks
WSB_2 = Infected white Shikabrown cocks

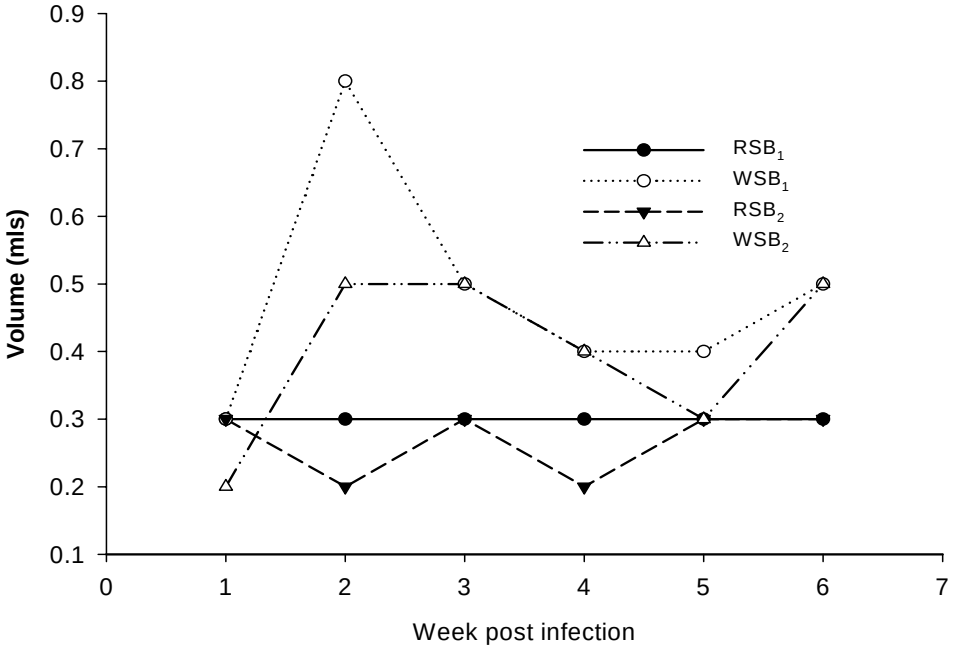

Fig. 4.3 Semen volume of control and infected Shikabrown cocks

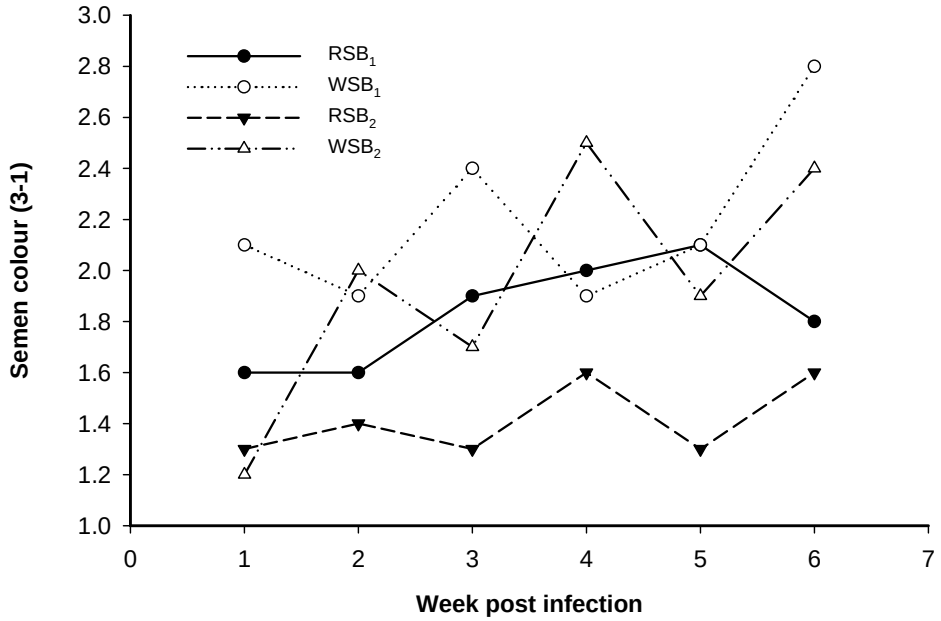

Fig. 4.4 Semen colour of control and infected Shikabrown cocks

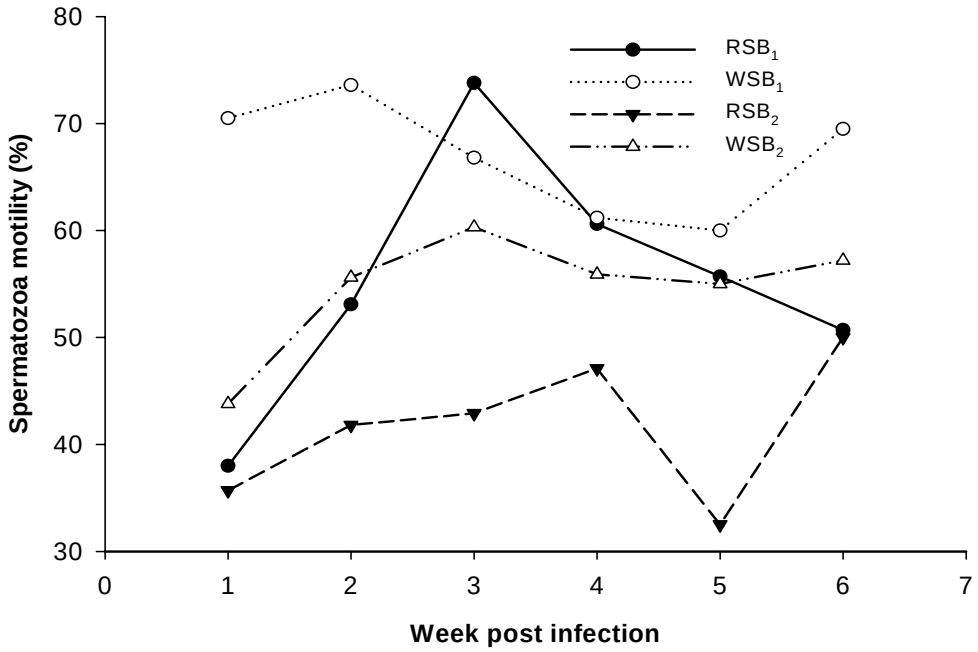

Fig. 4.5 Spermatozoa motility of control and infected Shikabrown cocks

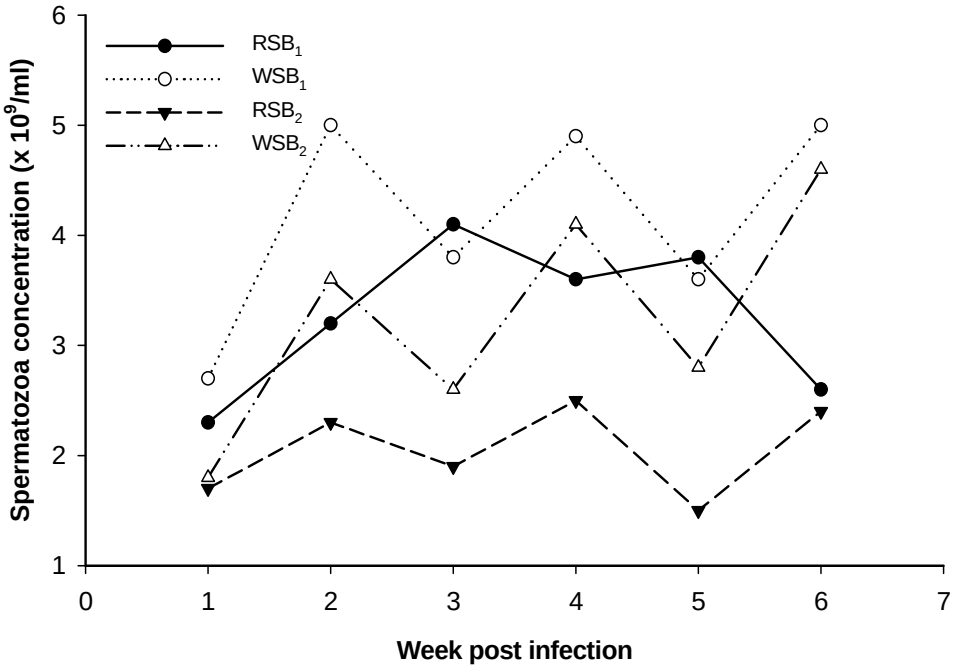

Fig. 4.6 Spermatozoa concentration of control and infected Shikabrown cocks

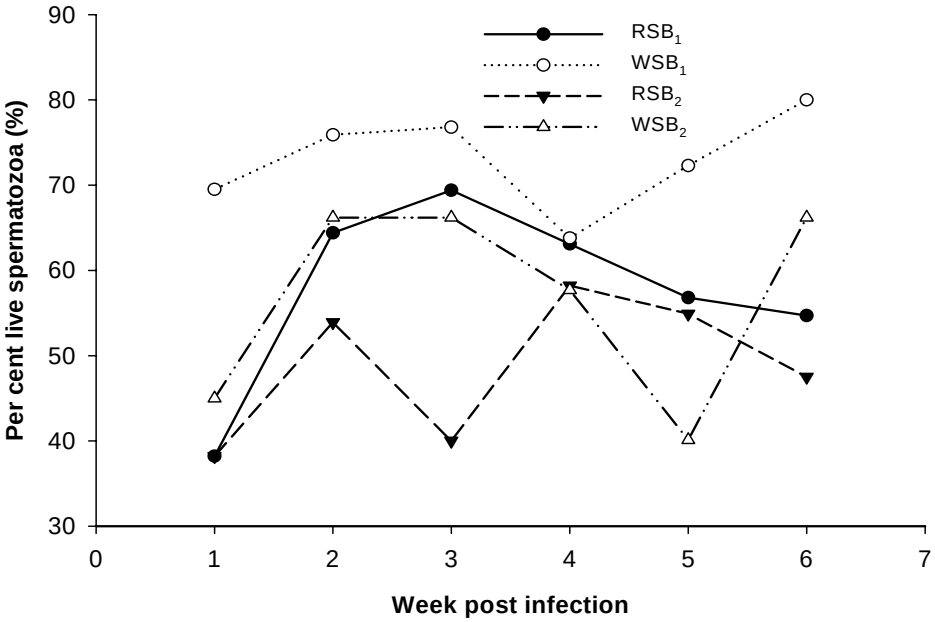

Fig. 4.7 Percentage live spermatozoa of control and infected Shikabrown cocks

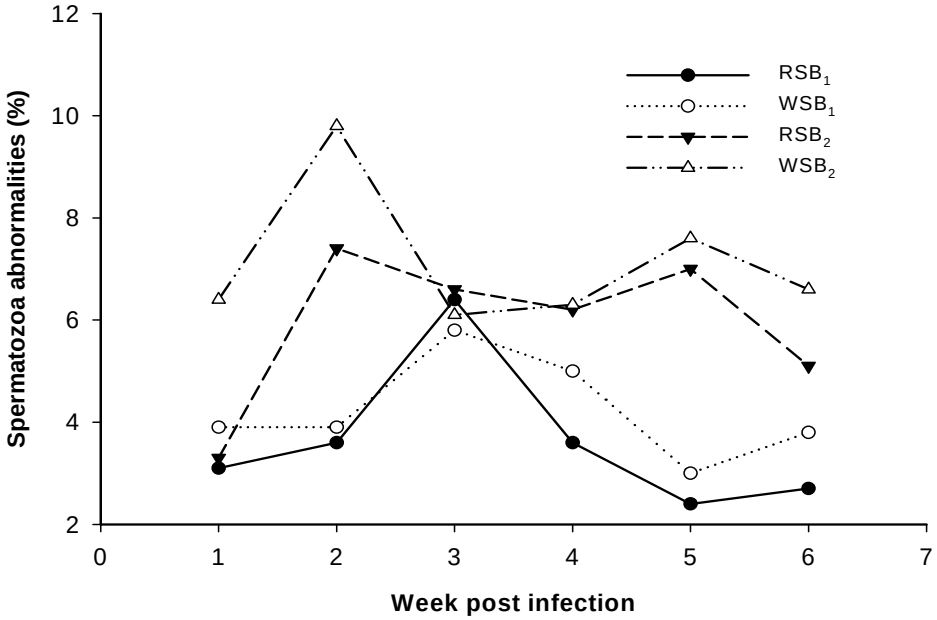

Fig. 4.8 Total sperm abnormalities of control and infectes Shikabrown cocks

4.5 DISCUSSION

The cloacal temperatures of the control red Shikabrown and white Shikabrown cocks were not significantly different (P>0.05). Similarly the cloacal temperatures of the infected red and white cocks were not significantly different (P>0.05). The red control cocks had slightly higher cloacal temperatures than the white control cocks. The cloacal temperatures of the infected red and white cocks were slightly higher than those of the control red and white cocks. This corroborates with the finding of Oladele (2004) who found an increase in cloacal temperatures of Shaver brown chicks vaccinated with Newcastle disease virus.

There was no significant difference (P>0.05) in the body weights of the control red Shikabrown and white Shikabrown cocks. Similarly there was no significant difference (P>0.05) in the body weight of infected red and white cocks, though the infected red cocks were slightly less heavier. This does not agree with the finding of Nwagu *et al.* (1996) who reported that the control red Shikabrown cocks were heavier than the control white Shikabrown cocks.

The infected red and white cocks were slightly less heavy in weight. This finding could be explained by the fact that the infected cocks eat less that the control cocks. This finding agrees with that of Okoye *et al*. (2000) who found that Newcastle disease infected chickens were significantly lighter in weight than the non-infected chickens at 2 and 3 weeks post infection. The chickens used by Okoye *et al*. (2000) were not vaccinated against Newcastle disease using the Kudu 113 strain of Newcastle disease virus and they used the VGF-1 strain of the virus.

The control and infected white Shikabrown cocks had higher semen volume throughout the period of the study than the red Shikabrown cocks. The control red cocks had higher semen volume than the infected cocks. The red Shikabrown cocks had better semen colour than the white Shikabrown cocks.

The white cocks had better spermatozoa motility than the red cocks, thus agreeing with the finding of Nwagu *et al*. (1999) who found that the white cocks had better spermatozoa motility than the red cocks.

The control white Shikabrown cocks had better spermatozoa concentration than the red cocks. The red cocks had higher antibody titres than the white cocks hence more protection from the Newcastle disease virus and its effects.

The high spermatozoa concentration of the white cocks obtained in this study agrees with the finding of Nwagu *et al*. (1999) who found a similar result working with the same strains of cocks.

The white Shikabrown cocks had higher per cent live spermatozoa than the red cocks. This shows that the white cocks had better semen quality than the red cocks. Infection reduced the per cent live spermatozoa of both the white and red cocks. The red cocks had higher antibody titres which gave them more protection from the Newcastle disease virus.

The control white cocks had higher percentage total spermatozoa abnormalities than the control red cocks. The infected red and white cocks had higher percentage total spermatozoa abnormalities than the control red and white cocks.

The percentage total spermatozoa abnormalities in this work ranged from 4.1% to 9.9% for the red Shikabrown cocks and 4.1% to 7.8% for the white Shikabrown cocks. These results differed from the findings of Omeje and Marire (1990) who reported the percentage total spermatozoa abnormalities of Rhode Island Red and Rhode Island White cocks to be 48% and 42% respsectively.

It can be concluded from this study that the control white cocks had better semen quality with respect to volume, motility, concentration and percent live spermatozoa than the control red cocks. The infected white cocks had lower semen concentration and total abnormalities than the infected red cocks.

In a Newcastle disease endemic environment like Zaria the cocks should be adequately protected from Newcastle disease through adequate and routine vaccination to prevent negative effects on semen quality.

CHAPTER 5

EFFECT OF A VELOGENIC NEWCASTLE DISEASE VIRUS ON TESTOSTERONE CONCENTRATION AND GONADAL SPERM RESERVES OF VACCINATED SHIKABROWN COCKS

5.1 ABSTRACT

Fifty 20 week old Shikabrown cocks were purchased from the National Animal Production Research Institute Shika. Five cocks out of the 50 consisting of three red Shikabrown and two white Shikabrown were bled for serum samples for testosterone assay at week 1, 3 and 6 pre- and post-infection. The blood samples were taken at thirty minutes interval for three hours for each cock on the day of sampling. The blood samples were centrifuged in a Hermle Z364 centrifuge at 251.6 g for 15 minutes and the sera stored in sera vials and kept in a deep freezer at −20°C until assayed using the Enzyme Linked Immunosorbent Assay technique. At the end of the experiment, twenty control and twenty infected cocks were slaughtered. Their testicles were removed, measured, minced and ground for the determination of gonadal sperm reserves.

It was found that the mean testosterone concentration of the control Shikabrown cocks had no particular trend. At week 6 the cocks had the highest mean testosterone concentration followed by at week 1; while at week 3 the cocks had the lowest mean testosterone concentration. The mean testosterone concentration post infection showed a decrease from week 1 to 6. The mean testosterone concentration peaks for the control red Shikabrown cocks at week 1 was 1 nmols/ml, week 3, 2 nmols/ml and week 6, 2.5 nmols/ml. The white Shikabrown cocks pre-infection had 12.5 nmols/ml

at week 1, 5.5 nmols/ml at week 3 and 3 nmols/ml at week 6 post infection respectively. The red Shikabrown cocks had mean testosterone concentration peaks of 9.7 nmols/ml, 6.3 nmols/ml and 2.7 nmols/ml at week 1, 3 and 6. The white cocks had 6.5 mean testosterone concentration peaks at week 1, 14.5 at week 3 and 6.5 at week 6.

The gonadal sperm reserves of the control red and white cocks were not significantly different but the gonadal sperm reserves of the control white cocks was significantly ($P<0.05$) higher than the gonadal sperm reserves of the infected red and white cocks. The total gonadal sperm reserve of the control white cocks was significantly ($P<0.05$) higher than the total gonadal sperm reserves of the infected red and white cocks. The weights of the left testicles of the control red and white cocks were significantly heavier than those of the infected red and white cocks.

5.2 INTRODUCTION

The general study of hormones such as gonadotropin-releasing hormones, follicle stimulating hormones, luteinizing hormones, estrogens, prostaglandins, testosterone and progesterone has clarified the fundamental mechanisms which regulate puberty, sexual (function) cycles, gestation and lactation in domestic animals (Edqvist *et al*., 1976; Peters and Ball, 1995; Rekwot, 2000). It is well known that adequate nutrition is an important management factor that affects sexual maturity, and improved feeding can optimize sperm cell production (Salisbury *et al*., 1978; Rekwot *et al*., 1987b; 1988). Testosterone concentration, spermiogram and other parameters increase in levels with increase in age and protein content of the diets (Rekwot *et al*., 1997), thus corroborating the works of Nolan *et al*. (1990), who reported that retarded testicular

growth and reduced testosterone concentrations were observed in bulls fed to gain weight slowly, compared with those fed to gain weight rapidly. Karg *et al*. (1976), reported very low levels of testosterone before puberty which rose sharply at the onset of puberty showing numerous pulsatile peaks as a function of time and age. Association between onset of spermatogenic activity and increased testosterone concentrations have been reported (Karg *et al*., 1976). Testosterone levels may not have a direct relationship with libido and semen quality in bulls (Post and Christensen, 1976).

Concentrations of serum testosterone increased linearly with advancing age in bulls (Lunstra *et al*., 1978; Rekwot *et al*., 1997). Circadian rhythms and well defined episodic peaks have been documented in serum testosterone profiles (Sanwal *et al*., 1974; Agarwal *et al*., 1983; Rekwot *et al*., 1997). The interaction of the brain- enzyme-environment with serum hormone effectiveness has been characterized more effectively in the avian brain than in any other vertebrate system (Ensley, 1976; Hutchinson and Steiner, 1983). In cockerels and other avian species, acute or long term stress reduces plasma luteinizing hormone and testosterone (Deviche, 1983). Wilson *et al*. (1979), reported changes in plasma testosterone levels in repeated samples drawn every 15 minutes. Since the dynamics of the changes were different in different individuals the authors attributed the changes to a pulsatile release of the hormones. Plasma testosterone levels in cockerels increase with the progress of sexual maturation (Sharp *et al*., 1977). Heiblum *et al*. (2000) in their experiments found that restrain increased plasma testosterone in intact roosters and failed to do so in castrated adults. It could be argued that the increase in testosterone in the restrained

birds is a result of augmentation in its secretion by the testis rather than a result of an increase in androgen production in the adrenal. Several mechanisms could be involved in the increase of testicular testosterone in response to stress (Heiblum *et al*., 2000). In red jungle fowl after aggressive encounters between pairs of males, testosterone levels increased in individuals that attacked first and decreased in their opponents (Johnson and Zuk, 1995). Heiblum *et al*. (2000), found that the greatest increase in testosterone in birds occurred during repeated blood sampling without restrain. There is a possible relationship between testosterone elevation during stress, aggressiveness and/or sexual behavior in domestic fowl.

Studies have shown that larger and heavier testes produced more spermatozoa than smaller and lighter ones (Coulter and Foote, 1979). Osinowo *et al*. (1981), reported that testicular size is highly correlated to seminiferous tubule diameter and gonadal sperm reserve in Bunaji bulls. Semen production was related to testicular development by a positive correlation between testicular width, gonadal and extragonadal sperm reserves and sperm concentration (Amann, 1970). Several workers have reported that scrotal circumference is a useful indicator of testicular development and subsequent sperm output especially in young bulls (Elmore *et al*., 1976).

Testosterone secretion from the testes of cocks is an integral part of the hormonal response to stress. The intense response of testosterone during blood sampling emphasizes the importance of minimizing stress during procedures for determination of androgens (Heiblum *et al*., 2000). Newcastle disease virus causes death and affects egg production and egg quality in the hen, but its effect on the cocks' reproductive

parameters including testosterone secretion and sperm reserve have not been properly documented.

The objective of this study was to determine the testosterone profiles and gonadal sperm reserves of the control and infected and non- Shikabrown cocks following challenge with a velogenic Kudu strain of the Newcastle disease virus.

5.3 MATERIALS AND METHODS

5.3.1 *Location*

The location of this study is as described in 3.3.1.

5.3.2 *Experimental cocks and management of cocks*

The experimental cocks and management cocks was as described in 3.3.2 and 3.3.3.

5.3.3 *Blood sampling*

Five cocks consisting of 3 red Shikabrown and 2 white Shikabrown were randomly chosen and bled for testosterone studies. These five cocks were among the 50 cocks sampled pre-infection in 3.3.4. Two ml of blood was taken from the wing vein of each of the cocks every 30 minutes for 3 h starting at 9 am on each day of sampling, at 6 weeks, 3 weeks and 1 week before infection. The same five cocks were among the twenty-five infected cocks. They were again sampled at 1 week, 3 weeks and 6 weeks after infection. The blood samples collected were centrifuged at 251.6 g for 15 minutes and the sera put in properly labelled plastic tubes and stored at -20°C in a deep freezer until assayed for testosterone.

5.3.4 Testosterone assay

A total of 200 sera samples were collected and stored as described in 5.3.3. Testosterone assay was done using the Enzyme Linked Immunosorbent Assay.

The microwell testosterone is a solid immunoassay utilizing the competitive binding principle. Testosterone present in serum sample competes with enzyme-labelled testosterone for binding sites on anti-testosterone antibody immobilized on the microwell surface. The testosterone kit was bought from the Dialab GMBH of Austria. The kit consisted of the following reagents and material (1) five calibrator standard reagents C_A, C_B, C_D, C_C and C_E. These standard reagents contained 0, 0.2, 1.0, 4.0 and 16.0 ng/ml of testosterone respectively and (2) enzyme conjugate which contained 12 ml testosterone-HRP conjugate; (3) microwell plate with 8 x 12 strips anti-testosterone Ig G adsorbed on microtitre plate; (4) substrate solution containing 12 ml of H_2O_2 – TMB 0.25 g/L; (5) stop solution which contained 12 ml of 0.15 mol/L of sulphuric acid.

Twenty five microliters (25 µl) of the serum samples were placed in each well of the microwell plate and twenty five microlitres (25 µl) of each of the five calibrator standard solutions were added to the serum samples in the microtitre plate well. One hundred microlitres (100 µl) of the enzyme conjugate was also added to each serum sample. The serum samples were properly mixed and incubated for 1 h at 37°C in an oven. After incubation the contents of the wells were decanted and the wells washed with three hundred microlitres (300 µl) of distilled water. Washing was repeated and the water was drained completely from the wells.

One hundred microlitres (100 µl) of substrate solution was added to each of the wells containing the sera samples and incubated at room temperature for 15 minutes in the dark. This resulted in a colour change (the solution turned blue). One hundred microlitres (100 µl) of stop solution was added to each of the wells containing the blue solution and it turned yellow. The microwell plate with the yellow solution was taken to the microwell reader (Multiskan Ascent produced by Thermo Electron Corporation of Finland) and the absorbance (E) read at 450 nm against blank. The sensitivity of the assay was 5 pg/ml and the inter and intra assay coefficients of variation were 3.9% and 6.2% respectively.

5.3.5 *Determination of gonadal sperm and spermatid reserves*

Out of the 50 Shikabrown cocks purchased from the National Animal Production Research Institute Shika, 25 cocks were infected with a velogenic NDV while 25 cocks served as control. At the expiry of the study 6 weeks post infection, 20 control cocks consisting of 11 Red Shikabrown and 9 white Shikabrown and 20 infected cocks consisting of 7 red Shikabrown and 13 white Shikabrown were weighed and slaughtered. The right and left testes were removed and their lengths and weights measured.

Gonadal sperm and spermatid reserves were determined as described by Igboeli and Rakha (1971) and Rekwot *et al*. (1994). Each testis was homogenized in 50 ml of physiological saline containing streptomycin sulphate (at 1 mg/ml) and sodium penicillin (G 100 I.U./ml) using a mortar and pestle. The volume of the homogenate was measured after rinsing the mortar and pestle with 20 ml of physiological saline and adding the effluent. Five milliliters of the homogenate were transferred to a conical

flask and diluted with 80 ml of saline. The diluted testicular homogenate sample was stored overnight in a refridgerator and filtered through a gauze and the volume of the filtrate measured. The number of spermatozoa and spermatid in the homogenate sample were determined using a hemocytometer. Both chambers of an improved Neubauer bright line homocytometer were filled with the testicular homogenate and spermatozoa were counted in five of the small centre boxes in both chambers. The total counts of both chambers were averaged and multiplied by 10^9 the resultant figure was recorded as spermatozoa per cu mm. Spermatozoa counted in the testicular homogenates included elongated spermatids and spermatozoa in stages vi, vii and viii of the seminiferous epithelium (Hafez, 1987).

5.3.6 *Statistical analysis*

The data on body weights, length and testicular weight, gonadal sperm and spermatid reserves were subjected to statistical analysis as in 3.3.7.

5.4 RESULTS

The mean testosterone concentration of the infected Shikabrown cocks shows a decrease from week 1 post infection to week 6 post infection (Fig. 5.1). The mean testosterone concentrations of the cocks pre-infection did not show a clear pattern. The mean testosterone concentration 6 weeks pre-infection was higher than that of week 3 and 1, with week 3 mean testosterone concentration being the lowest (Fig. 5.1). The mean testosterone concentration of the infected cocks shows that the velogenic Newcastle disease virus affected the testosterone secretion of the cocks. The challenge of the cocks with the velogenic Newcastle disease stressed the cocks leading to increased production of

corticosteroids which affected the interstitial cells which produce testosterone in the testicles. The virus used in this experiment is velogenic and pantropic therefore it is likely that it has adversely affected the interstitial cells responsible for the production of testosterone thus the decrease in testosterone concentration in infected cocks.

The mean of the lowest testosterone concentration values observed during the 3 h sampling period were taken as basal testosterone concentration. A peak was defined as each single value or series of values 2-fold above mean basal concentration (Renaville *et al.*, 1983). The mean number of testosterone concentration peaks of the control red Shikabrown cocks increased from week 1 to 6. In the white Shikabrown cocks the reverse was the case, the mean testosterone concentration peaks decreased from week 1 to 6. The pre-infection mean testosterone concentration peaks of all the cocks (red and white) was about the same value for week 1 and 6 while week 3 was lower in value than both of them (Figs. 5.2 and 5.3).

The mean number of testosterone concentration peaks for the red cocks post infection showed a decrease in the number of peaks from week 1 to 6. In the white cocks week 3 had the highest mean testosterone concentration peaks while the value for week 1 and 6 was the same. All the cocks (red and white) post infection did not show a clear pattern in the mean number of testosterone concentration peaks. Week 1 and 3 had high values compared to a low value for week 6 (Figs. 5.2 and 5.3).

There was no significant difference ($P>0.05$) in the mean body weight and right testis weight of both control and infected cocks. There was no significant difference ($P>0.05$) in

the left testis weight of the control red and white cocks, similarly the left testis weight of the infected red and white cocks was not significantly different. The left testis weight of the control red and white cocks was significantly different ($P<0.05$) from that of the infected red and white cocks (Table 5.1).

The gonadal sperm reserves of the right testes of the control red and white cocks was not significantly different, but the gonadal sperm reserve of the control white cocks was significantly higher than that of the infected white and red cocks (Table 5.1). The gonadal sperm reserve of the left testes was not significantly different ($P>0.05$) for both the control and infected red and white cocks (Table 5.1).

The total gonadal sperm reserve of the control white cocks was significantly higher ($P<0.05$) than that of the infected red and white cocks.

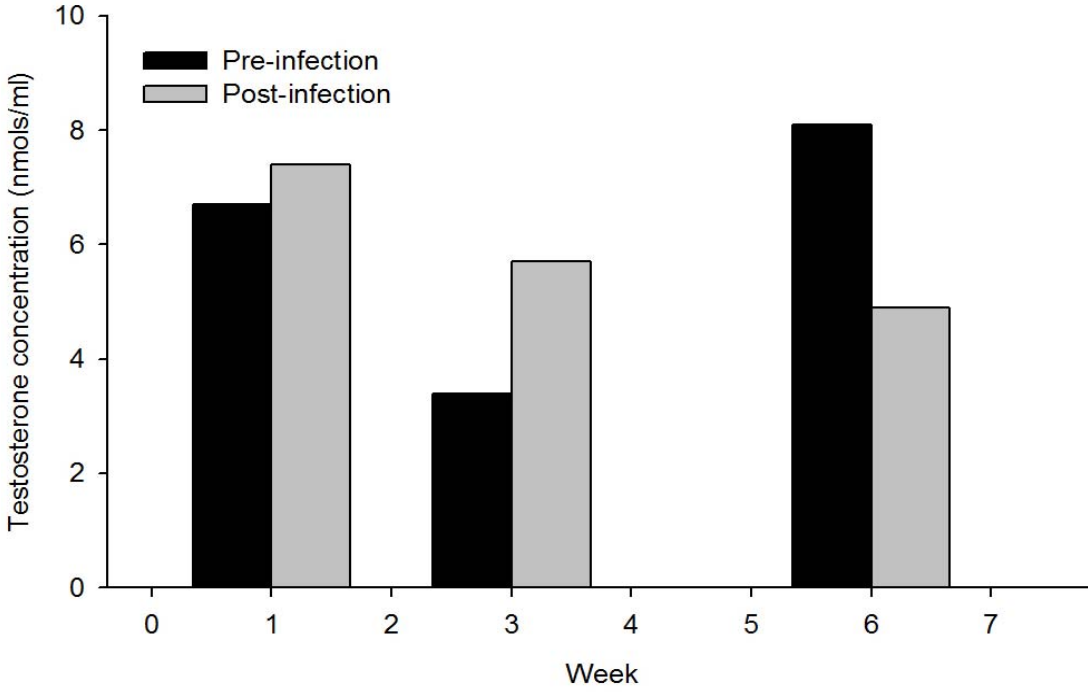

Fig. 5.1. Mean testosterone concentration of Shikabrown cocks pre and post infection

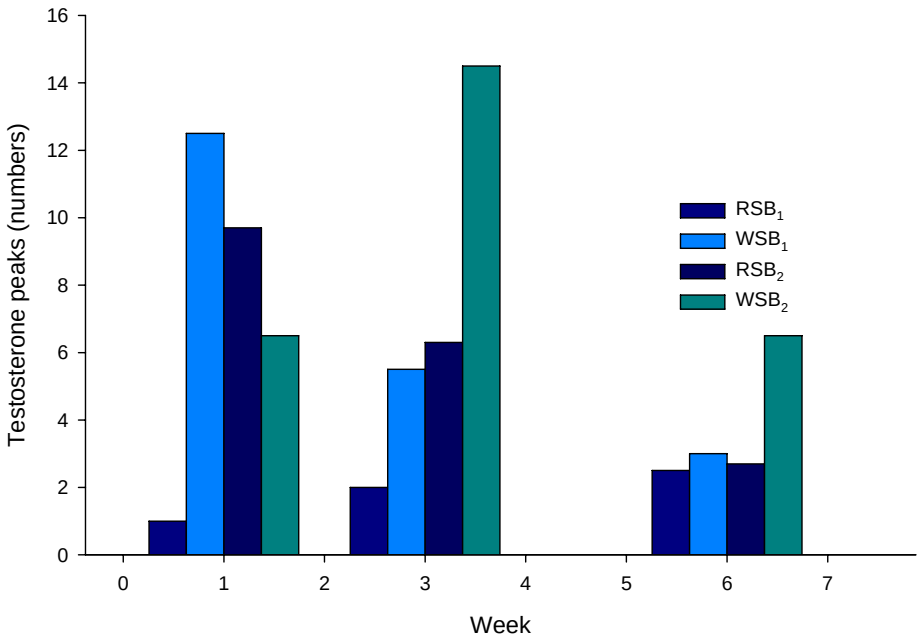

Fig. 5.2 Mean number of testosterone peaks of Shika brown cocks

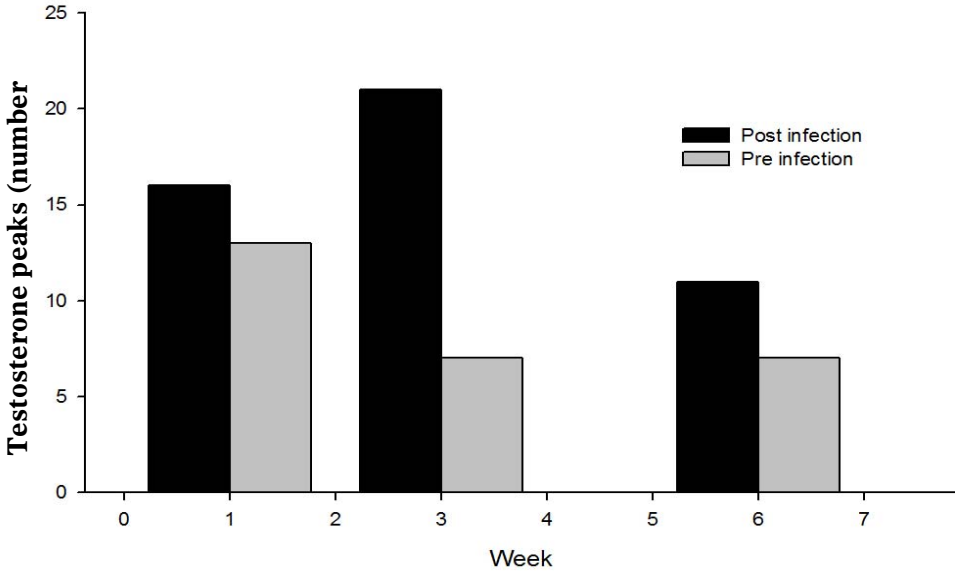

Fig. 5.3. Mean number of testosterone peaks of Shikabrown cocks pre and post infection

Table 5.1. Gonadal sperm and spermatid reserves of control and infected red Shikabrown and white Shika brown cocks

	Control		Infected	
Parameters	RSB (n = 11)	WSB (n = 9)	RSB (n=7)	WSB (n=13)
Body weight (kg)	2.4±0.2[a]	2.4±0.1[c]	2.1±0.4[b]	2.2±0.1[d]
Weight of right testis (g)	12.7±2.4	12.1±2.9	12.5±2.3	12.0±2.3
Weight of left testis (g)	13.7±2.4[a]	13.3±1.8[c]	11.5±4.9[a]	11.5±5.0[d]
Length of right testis (cm)	4.6±0.4	4.4±0.5	4.7±0.2	4.7±0.5
Length of left testis (cm)	4.8±0.4	4.6±0.6	4.9±0.1	4.8±0.3
Gonadal sperm reserves (x 10^9 g/testis)				
Gonadal sperm reserve of right testes	1.7±0.2	2.1±0.2	1.5±0.2[d]	1.7±0.6[d]
Conadal sperm reserve of left testes	1.7±0.2	2.1±0.3	1.6±0.3	2.0±0.5
Total gonadal sperm and spermatid reserves	3.4±0.2	4.2±0.3[e]	3.1±0.4[f]	3.2±0.3[f]

abcdef = Means ± S.D. within rows with different letter superscripts are significantly different (P<0.05).

5.5 DISCUSSION

The presence of basal and peak testosterone concentrations agreed with the reports of Sanwal *et al*. (1974), in exotic bulls and Rekwot *et al*. (1997) in Zebu bulls. The testosterone profiles in this study were episodic, pulsatile or temporal in nature. This confirms earlier report in bulls by Thibier (1976) and Agarwal *et al*. (1983). The testosterone episodic peaks observed in this study agreed with earlier observations of Sanwal *et al*. (1974); Agarwal *et al*. (1983) and Rekwot *et al*. (1997) in bulls.

The exact significance of these testosterone episodic peaks is not clear but may be related to the sexual and behavioural states of animals to photoperiodicity, temperatures and postural states of the animal (Sanwal *et al*., 1974).

According to Karg *et al*. (1976) and Rekwot *et al*. (1997) the higher the testosterone concentration the better the semen characteristics. In the present study the control white Shikabrown cocks had higher mean testosterone concentration peaks than the control red Shikabrown cocks. The mean testosterone concentration peaks of the infected red and white cocks did not follow any clear pattern. The infected red cocks had high mean testosterone concentration peaks at weeks 1 and 3 while the infected white cocks had high mean testosterone concentration peaks at weeks 3, and the same mean testosterone concentration peaks at week 1 and 6.

The mean testosterone concentration of Shikabrown cocks post infection shows a decline from week 1 to 6, indicating that the velogenic Newcastle virus affected testosterone production.

In the cock testosterone also plays a major role in the onset of puberty such as development of genital tract, libido, initiation and potentiation of spermatogenesis in conjection with androgen building proteins and follicle stimulating hormone (Etches, 1996; Hafez, 1987 and 1990). This study showed that there was a decrease in the mean testosterone concentration of infected cocks due to the stress of infection hence a delay in the onset of puberty in cocks infected with Newcastle disease. Daily sperm production and daily production per gram of parenchyma of testis have been estimated in dairy and beef *Bos taurus* bulls (Almquist and Amann, 1961; Weisgold and Almquist, 1979) in several breeds of Bos indicus as well as in crossbreed Bos indicus (Wildeus and Entwistle, 1982; Tegegne *et al*., 1992).

The control white Shikabrown cocks had higher semen volume and spermatozoa concentration, this can be corroborated by the high gonadal sperm reserves in the control white Shikabrown cocks in this work. Reports have shown strong association between plane of nutrition, body weight and scrotal growth (van DeMark and Mauger, 1964; Reeves and Johnson, 1976; Rekwot *et al*., 1988). The differences in the right and left testicular weight in the two breeds in the present study is consistent with previous studies in cattle (Fields *et al*., 1979; 1982; Entwistle, 1983; Tegegne *et al*., 1992, 1994). As it is generally in most animal species and poultry the left testis is always heavier than the right (Roberts, 1971; Etches, 1996). This study confirmed the earlier studies (Etches, 1996).

The Newcastle disease virus causes decrease in testosterone concentration and gonadal sperm reserves in Shikabrown cocks.

Since the velogenic Newcastle disease virus caused a decrease in the mean testosterone concentration of the Shikabrown cocks they should be adequately and routinely vaccinated against Newcastle disease.

CHAPTER 6

EFFECT OF A VELOGENIC NEWCASTLE DISEASE VIRUS ON BODY AND ORGAN WEIGHTS OF VACCINATED SHIKABROWN COCKS

6.1 ABSTRACT

Fourty Shikabrown cocks consisting of twenty control and twenty infected cocks were slaughtered at the age of thirty-two weeks after infection with a velogenic Newcastle disease virus. They were weighed before being sacrificed and their internal organs were removed and weighed. The organs involved were the right and left testicles, liver, spleen, thymus, heart, bursa of Fabricius, brain and adrenal glands. Testicular lengths were taken. Sections of these organs were taken and stored in Bouin's solution for 24 h and sent for histology.

The liver, spleen, thymus, heart and adrenal glands of the infected red Shikabrown cocks were heavier than those of the control red Shikabrown and white cocks and infected white Shikabrown cocks. The weight of the spleen of both the control and infected red and white cocks was not significantly different ($P>0.05$). There was no significant difference in the weight of the brain of both control and infected red and white cocks. The bursal weight of the control white cocks was heavier than that of the control red and infected red and white Shikabrown cocks. The infected red cocks had heavier adrenal glands than the control red and infected red and white cocks. The infected red cocks had a higher liver body weight ratio than the control red and white cocks and infected white cocks. The spleen body weight ratio was the same for both control and infected red and white cocks. The control white cocks had the least

thymus body weight ratio. The infected red cocks had the highest heart body weight ratio. The brain body weight ratio was the same for both control and infected red and white cocks. The control white cocks had a higher bursa body weight ratio than the infected red and white cocks and the control red cocks. The infected red cocks had the highest adrenal bodyweight ratio. Only one infected white cock showed perivascular infiltration of lymphocytes and foci of glial cells.

The increase in organ weights was seen mostly in the infected red cocks. The infected red cocks had slightly higher antibody titres than the infected white cocks, this gave the red cocks more protection from the velogenic Newcastle disease virus and hence the virus had little or no effect on the growth of the organs. It is recommended that chickens should be routinely and adequately vaccinated against Newcastle disease to prevent atrophy of the bursa of Fabricius the primary organ responsible for immunity in birds.

6.2 INTRODUCTION

Growth and development of various organs of the domestic fowl have been of interest since the initial domestication of *Gallus gallus* (Thaxton, 2002). The gross lesions and the organs affected in birds infected with NDV are dependent on the strain and pathotype of the infecting virus in addition to the host and all the other factors that may affect the severity of the Newcastle disease (Alexander, 1988). There are no pathognomonic lesions associated with any form of the disease. Gross lesions may also be absent in ND infected birds (Alexander, 1988). In the chicken hemorrhagic lesions are observed in the small intestines, proventriculus and ceca. They are markedly hemorrhagic and appear to result from necrosis on the intestinal

wall or lymphoid foci such as cecal tonsils. Generally gross lesions are not observed in the central nervous system of birds infected with NDV regardless of pathotype (McFerran and McCracken, 1988). Chickens and turkeys infected in lay with velogenic viruses usually reveal egg yolk in the abdominal cavity and the ovarian follicles are often flaccid and degenerative. Hemorrhage and discoloration of the other reproductive organs may however, occur (Alexander, 1988).

Histopathologic changes resulting from NDV infection in the female reproductive tract are extremely variable. Biswal and Morrill (1954) reported that the greatest functional damage by Newcastle disease virus was to the uterus or shell gland of the oviduct. Atresia of follicles with infiltration of inflammatory cells and formation of lymphoid aggregates were seen. Small focal areas of necrosis in the liver and sometimes with hemorrhage in the gall bladder and heart have been reported (Alexander, 1988). Lymphocyte infiltration has been reported in the pancreas while congestion and petechial hemorrhages of the comb and wattles are common. In the central nervous system, a non-purulent encephalomyelitis with neuronal degeneration, foci of glial cells, perivascular infiltration of lymphocytes and proliferation of endothelial cells have been observed (Stevens *et al.,* 1976; McFerran and McCracken, 1988).

The adult avian spleen has been characterized as predominantly a lymphocyte producing and an erythrocyte-destroying organ (Hodges, 1974). Lucas and Jamroz (1961) also characterized the spleen during the early neonatal period as a lymphocytogenic and monocytogenic organ.

Central to the immune response is the normal embryonic development of thymic-derived lymphocytes (T-cells) and bursal or bone marrow derived lymphocytes (Glick, 1977). While the chicks bursa may accept stem cells by day eight the ability of the bursal lymphocytes to produce IgM occurs at approximately day 13 (Kinkade and Cooper, 1971). The adrenal, thyroid, liver and the gastrointestinal tract are sensitive to heat stress (Selye, 1950). The adrenal cortex enlarges 6 to 24 hours after initiation of a severe heat stress (Engel *et al*., 1943 and Ingle, 1938). Thyroid size in chicken varies considerably with season, environmental temperature, diet, age and functional state (Sturkie, 1954).

Newcastle disease causes marked hemorrhage in the intestinal wall, ceca and proventriculus due to necrosis of the intestinal wall or cecal tonsils (Hanson *et al*., 1973).

Generally gross lesions are not observed in the central nervous system of birds infected with Newcastle disease virus regardless of the pathotype (McFerran and McCracken, 1988). Gross pathologic changes are not usually present in the respiratory tract but when observed consist of hemorrhagic lesions and marked congestion of the trachea (Alexander and Allan, 1974). In infected chickens and turkeys the ovarian follicles are often flaccid and degenerative. Hemorrhage and discolouration of the other reproductive organs may occur (Alexander and Allan, 1974).

The objective of this study was to evaluate the effect of the velogenic Kudu strain Newcastle disease virus on the body and organs of vaccinated Shikabrown cocks.

6.3 MATERIALS AND METHODS

6.3.1 *Location*

The location of this study is as described in section 3.3.1.

6.3.2 *Experimental cocks*

The experimental cocks were managed as described in 3.3.2.

6.3.3 *Morphometric and biometric studies*

Fourty cocks consisting of 20 control and 20 infected Shikabrown cocks aged 32 weeks were used. They were weighed, slaughtered, their organs were dissected out and weighed. Their livers, spleens, thymus, hearts, bursae of fabricius, brains and adrenal glands were dissected out and weighed. The organ body weight ratios were obtained by dividing the organ weights in grams by the body weight in grams and multiplying the products by 1000 (Krasselt, 1986).

Samples of these organs were fixed in bouins solution for 24 h after which a little cut section of the organ specimen with its label included in an enclosed metallic or plastic capsule was washed several times in tap water, this was done to remove deposits of the fixative so as to reduce artifacts. The little cut section of the organ specimen enclosed in the metallic or plastic capsule with its label was passed through ascending grades of ethanol or any dehydrating agent, starting from 70% to 80% to 95% and to 100%. The specimen spent 2 h each in the 70% and 80% alcohol and 1 hour each in the 95% and 100% alcohol. After the dehydration of the specimen in alcohol it was then transferred into a clearing agent, xylene for 2 hours or more depending on the size of the tissue. When this was complete the specimen was then impregnated with

wax. The wax penetrates into the tissue displacing the xylene. The capsule containing the tissue is transferred into molted wax and left for 2 – 4 h in an oven at 50°C - 60°C, after which the tissue was embedded. This is the process of blocking the tissue in molten wax and allowed to solidify. After embedding, the blocked tissue was mounted on a wooden or plastic block and left in the deep freezer to freeze. The blocked tissue was then sectioned using the microtome into thin slides of tissue about 5 – 15 microns. The cut sections were spread out in a water bath and picked up with slides which had been smeared with adhesive, glycerine/albumin mixture. The picked up sections were allowed to dry in an oven set at 56°C for 1 h after which they were stained.

6.4 RESULTS

Table 6.1 shows no significant difference in the body weights of the control and infected red and white Shikabrown cocks. The weight of the liver in the control and infected red cocks was not significantly different ($P>0.05$). The weight of the control and infected white cocks were also not significantly different ($P>0.05$), but the weight of the liver in the infected red cocks was significantly heavier than that of the control and infected white cocks (Table 6.1). The weight of the spleen of both the control and infected red and white cocks was not significantly different ($P>0.05$). The thymus weight of the infected red cocks was significantly heavier ($P<0.05$) than that of the control red and white cocks (Table 6.1). The heart weight of the infected red cocks was significantly ($P<0.05$) heavier than the control red and white cocks and infected white cocks. There was no significant difference ($P>0.05$) in the weight of the brain of both control and infected red and white cocks. The bursal weight of the control white

cocks was heavier than that of the control red and infected red and white Shikabrown cocks. The infected red cocks had heavier adrenal glands than the control red and infected red and white cocks (Table 6.1).

Table 6.2 shows the organ body weight ratios of the red and white cocks. The infected red cocks had a higher liver body weight ratio than the control red and white cocks and infected white cocks. The spleen body weight ratio was the same for both control and infected red and white cocks. The control white cocks had the least thymus body weight ratio. The infected red cocks had the highest heart bodyweight ratio. The brain body weight ratio was the same for both control and infected red and white cocks. The control white cocks had a higher bursa body weight ratio than the infected red and white cocks and the control red cocks. The infected red cocks had the highest adrenal body weight ratio.

The histopathology of NDV infections is as varied as the clinical signs and gross lesions and can be greatly affected by the same parameters (Beard and Hanson, 1984). Regressive changes found in the lymphopoetic system consist of disappearance of lymphoid tissue. There is hyperplasia of the reticulo histiocytic cells in various organs especially the liver. Necrotic lesions were found throughout the spleen. Focal vacuolation and destruction of lymphocytes were seen in the cortical areas and germinal centres of the spleen and thymus (Beard and Hanson, 1984). Marked degeneration of the medullary region was seen in the bursa.

None of the infected cocks showed the changes enumerated above histologically. Only one infected white Shikabrown cock showed a brain lesion of non-purulent

encephalomyelitis with neuronal degeneration, foci of glial cells and perivascular infiltration of lymphocytes.

Table 6.1. Mean body and organs weights of sacrificed control and infected Shikabrown cocks

Parameter	Control RSB_1	Infected RSB_2	Control WSB_1	Infected WSB_2
Body weight (kg)	2.4±0.1[a]	2.1±0.1[a]	2.4±0.1[a]	2.2±0.1[a]
Liver (g)	25.6±1.1[a]	27.0±0.2[a]	24.8±0.2[b]	2.4±1.0[b]
Spleen (g)	2.9±0.2	3.1±0.3	2.7±0.2	2.4±0.2
Thymus (g)	3.8±0.5	5.0±0.6	3.4±0.6	4.2±0.4
Heart (g)	12.8±0.6[a]	15.5±0.7[b]	12.4±0.7[a]	12.8±0.1[a]
Brain (g)	2.9±0.1	2.9±0.2	2.5±0.2	3.0±0.1
Bursa (g)	0.6±0.1[a]	0.5±0.1[a]	0.8±0.1[b]	0.6±0.1[a]
Adrenal gland	0.4±0.1	0.5±0.1	0.3±0.1	0.4±0.0

abcdefgh = Means ± SD within rows with different letter superscripts are significantly different ($P<0.05$).

Key

RSB_1 = Control red Shikabrown cocks
RSB_2 = Infected red Shikabrown cocks
WSB_1 = Control white Shikabrown cocks
WSB_2 = Infected white Shikabrown cocks

Table 6.2 Organ body weight ratios of Shikabrown cocks infected with a velogenic Newcastle disease virus

Parameter	RSB_1	RSB_2	WSB_1	WSB_2
Liver	11.00	13.00	10.00	12.00
Spleen	1.00	1.00	1.00	1.00
Thymus	2.00	2.00	1.00	2.00
Heart	5.00	7.00	5.00	6.00
Brain	1.00	1.00	1.00	1.00
Bursa	0.20	0.24	0.30	0.28
Adrenal gland	0.16	0.24	0.14	0.18

Key

RSB_1 = Control red Shikabrown cocks
RSB_2 = Infected red Shikabrown cocks
WSB_1 = Control white Shikabrown cocks
WSB_2 = Infected white Shikabrown cocks

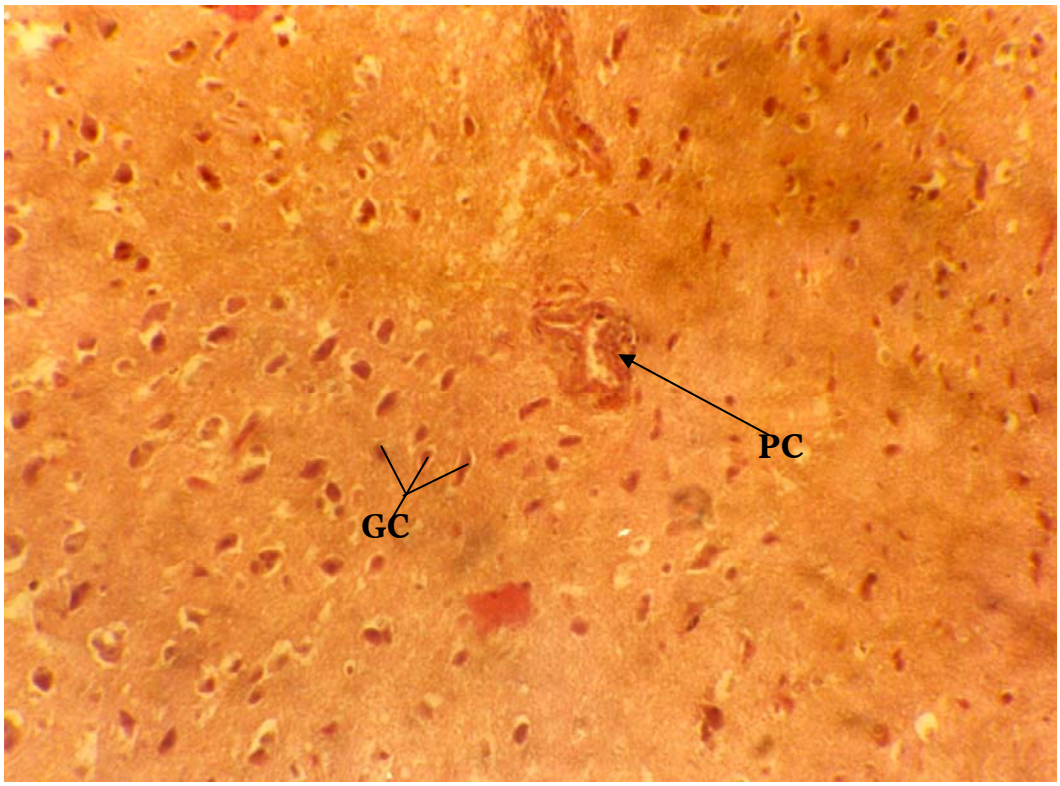

Plate VI. Perivascular cuffing and gliosis in white Shikabrown infected cock No. 001

PC = Perivascular cuffing
GC = Glial cells

6.5 DISCUSSION

In the present study the heart weights of the infected red cocks differed significantly from those of the control red and white cocks and this disagrees with work of Panwitz and Berg (1998), who reported non-significant heart weight changes in turkeys. Rajpal *et al*. (1992, 1993) and Das *et al*. (1997), found that the average gross weight of the duck's heart was heavier than that of the fowl. The bursa of Fabricius of the cocks in this study decreased in weight contrary to the findings of Dafwang *et al*. (1985), who reported that chicks fed antibiotics had heavier bursae. The bursae control immunity, heavier bursae translate to enhanced immunity for the cocks.

The decrease seen in the weight of the bursa of Fabricius is an indication that the velogenic Newcastle disease virus used in this study may cause immunosuppression in infected cocks and this corroborates the findings of Okoye *et al*. (2000) who reported atrophy of the bursa of Fabricius in Newcastle disease infected chickens. Relative heart weight increased significantly in the infected red cocks used in this work. Perivascular cuffing seen in this work agrees with what has been reported by Hanson (1978), Beard and Hanson (1984) and Alexander *et al*. (1985), who reported a non-purulent encephalomyelitis with neuronal degeneration foci of glial cells and perivascular infiltration of lymphocytes in Newcastle disease infected chickens. The implication of the lesions seen in the brain of ND virus infected vaccinated cocks is that the virus could cause brain lesions without death and nervous signs and such lesions may last up to 6 weeks post infection.

The Shikabrown cocks used in this study were previously vaccinated with Newcastle disease virus vaccine. Their subsequent challenge with the velogenic Newcastle disease virus led to the production of more antibodies against the velogenic Newcastle disease virus.

It could be concluded from the study that the higher antibody titres seen in the infected red cocks gave them more protection from the velogenic Newcastle disease virus. This is because the Newcastle disease virus had little or no detrimental effect on their organs.

It is recommended that poultry farmers should routinely and adequately vaccinate their chickens to protect them from Newcastle disease infection.

CHAPTER 7

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

This work has shown clearly that the mean antibody titre of 1.9±0.7 $\log_2$ to 4.6±0.4 $\log_2$ provided protection to the red and white cocks against the velogenic Newcastle disease virus. It was clearly seen that the packed cell volume of the red Shikabrown cocks was slightly higher than that of the white Shikabrown cocks because they had higher mean antibody titres and withstood the challenge with the Newcastle disease virus better than the white Shikabrown cocks. The red Shikabrown cocks reacted better to vaccination than the white Shikabrown cocks. The slightly higher antibody titre in the red Shikabrown cocks after challenge is an indication that they responded better to previous vaccination against Newcastle disease; based on this finding it was expected that the red Shikabrown cocks will better resist infection by the Newcastle disease virus than the white Shikabrown cocks. This speculation has been strengthened by the fact that the changes in the mean packed cell volume of the red Shikabrown cocks was not affected as much as that of the white Shikabrown cocks after challenge with the velogenic Newcastle disease virus. From this study hemagglutination inhibition Newcastle disease antibody titres of as low as 2 $\log_2$ were able to protect birds challenged with the velogenic Newcastle disease virus used. This value was below the recommended value of 5 $\log_2$ (Allan *et al*., 1978).

It is therefore possible that birds with antibody titres of less than 5 $\log_2$ will be protected when exposed to a velogenic Newcastle disease virus. This study also confirmed that vaccinated birds could be infected with velogenic Newcastle disease virus when exposed. The challenge of the red Shikabrown and white Shikabrown cocks with the velogenic Kudu 113 strain of Newcastle disease virus in this study led to an increase in antibody titres in the cocks.

Based on the results of semen parameters it can be concluded that the white Shikabrown cocks had better semen quality than the red Shikabrown cocks. In addition the non-infected cocks had better semen quality than the Newcastle disease virus infected cocks.

It was observed in this study that the velogenic Newcastle disease virus caused a decrease in testosterone concentration and gonadal sperm reserves in Shikabrown cocks.

The implications of the lesions seen in the brain of Newcastle disease virus infected vaccinated cocks is that the virus could cause brain lesions without death and nervous signs and such lesions may last up to 6 weeks post infection for none of the infected cocks died.

To ascertain adequately whether the various abnormalities observed in infected cocks can be translated into infertility in breeder hens further breeding studies need to be conducted. The white Shikabrown cocks had better semen qualities than the red Shikabrown cocks and therefore the white Shikabrown cocks should be used for breeding purposes.

In a Newcastle disease endemic environment such as Zaria farmers keeping red and white Shikabrown chickens should adequately and routinely vaccinate against Newcastle disease. It is also recommended that Shikabrown cocks should be routinely re-vaccinated against Newcastle disease to ensure that the level of antibodies is high enough to prevent adverse effect on their semen quality.

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APPENDICES

Appendix 1: ANOVA on hemagglutination inhibition antibody titre (HIT) of Shikabrown breeder cocks pre-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	118.3866667	23.6773333	7.71	0.0001
Breed	1	3.0040184	3.0040184	0.98	0.3235
Week * Breed	5	2.4673934	0.4934787	0.16	0.9766

T-test on means of HIT of Shikabrown cocks pre-infection

T-grouping	Mean	No. of cocks	Week
A	4.120	50	2
A	4.080	50	3
A	3.540	50	1
A	3.540	50	4
B	2.620	50	6
B	2.540	50	5

T-test on means of HIT of the strains of Shikabrown cocks pre-infection

T-grouping	Mean	No. of observations	Strain
A	3.519	133	RSB
A	3.317	167	WSB

Appendix 2: ANOVA on hemagglutination inhibition antibody titre (HIT) of Shikabrown breeder cocks post infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	307.8400000	61.568000	26.76	0.0001
TRT	1	1689.813333	1689.813333	34.36	0.0001
Breed	1	72.566255	72.566255	31.54	0.0001
Week * TRT	5	46.586667	9.3173333	4.05	0.0015
Week * Breed	5	3.993874	0.798775	0.35	0.8839
TRT*Breed	1	0.000000	0.000000	0.00	1.0000

T-test for HIT means for Shikabrown cocks six weeks post infection

T-grouping	Mean	No. of cocks	Week
A	6.700	50	2
B	5.940	50	1
B	5.460	50	4
B	5.400	50	3
C	5.040	50	5
D	3.380	50	6

T-test for HIT means of control and infected Shikabrown cocks, six weeks post infection

T-grouping	Mean	No. of observations	TRT
A	7.693	150	Infected
B	2.947	150	Control

T-test on means of HIT of strains of Shikabrown cocks 6 weeks post infection

T-grouping	Mean	No. of observations	Strain
A	5.756	168	WSB
B	4.705	132	RSB

Appendix 3: ANOVA and T-test on packed cell volume (PCV) of RSB breeder cocks pre-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	2984.350000	596.870000	29.97	0.0001
Breed	1	266.134846	266.134846	13.36	0.0003
Week * Breed	5	39.889012	7.977802	0.40	0.8482

T-test on means of PCV means of Shikabrown pre-infection

T-grouping	Mean	No. of cocks	Week
A	42.280	50	6
B	40.200	50	5
B	39.620	50	4
B	38.500	50	3
C	35.080	50	1
D	32.980	50	2

T-test on means of PCV of strains of Shikabrown cocks pre-infection

T-grouping	Mean	No. of observations	Strain
A	39.165	133	RSB
A	37.269	167	WSB

Appendix 4: ANOVA on packed cell volume (PCV) of Shikabrown breeder cocks post infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	427.4666667	85.4933333	6.24	0.0001
TRT	1	246.6133333	246.6133333	18.00	0.0001
Breed	1	4.6801948	4.6801948	0.34	0.5593
Week * TRT	5	112.586667	22.5173333	1.64	0.1484
Week * Breed	5	190.4321429	38.0864286	2.78	0.8181
TRT*Breed	1	51.8364693	51.8364693	3.78	0.0527

T-test on means of PCV of Shikabrown cocks post-infection

T-grouping	Mean	No. of cocks	Week
A	43.400	50	5
A	43.320	50	4
C	41.640	50	3
C	41.480	50	6
C	40.800	50	1
D	39.560	50	2

T-test on means of PCV of control and infected Shikabrown post-infection

T-grouping	Mean	No. of observations	TRT
A	42.440	150	Control
B	40.627	150	Infected

T-test on means of PCV of strains of Shikabrown cocks post-infection

T-grouping	Mean	No. of observations	Strain
A	41.674	132	RSB
A	41.423	132	WSB

Appendix 5: ANOVA on total proteins of Shikabrown breeder cocks pre-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	3.40466667	0.68093333	2.79	0.0177
Breed	1	0.7879994	0.7879994	3.23	0.0935
Week * Breed	5	0.35038419	0.07007684	0.29	0.9200

T-test on means of total protein of Shikabrown cocks pre-infection

T-grouping	Mean	No. of cocks	Week
A	4.5880	50	6
A	4.5660	50	4
A	4.5020	50	3
A	4.4220	50	1
C	4.3760	50	2
C	4.2860	50	5

T-test on means of total protein of strains Shikabrown cocks pre-infection

T-grouping	Mean	No. of observations	Breed
A	4.5024	167	WSB
A	4.3992	133	RSB

Appendix 6: ANOVA on total protein of Shikabrown breeder cocks post infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	2.86536667	0.57307333	2.21	0.0536
TRT	1	0.02083333	0.02083333	0.08	0.7771
Breed	1	0.59645022	0.59645022	2.30	0.1305
Week * TRT	5	1.29896667	0.25979333	1.00	0.4171
Week * Breed	5	1.55734978	0.31146996	1.20	0.3089
TRT*Breed	1	0.00276929	0.00276929	0.01	0.9178

T-test on means of total protein of Shikabrown cocks post infection

T-grouping	Mean	No. of cocks	Week
A	4.796	50	6
A	4.732	50	5
A	4.730	50	3
A	4.678	50	4
A	4.648	50	1
B	4.486	50	2

T-test on means of total protein of control and infected Shikabrown cocks post infection

T-grouping	Mean	No. of observations	TRT
A	4.6867	150	Infected
A	4.6700	150	Control

T-test on means of total protein of strains of Shikabrown cocks post infection

T-grouping	Mean	No. of observations	Strain
A	4.7179	168	WSB
A	4.6280	132	RSB

Appendix 7: Analysis of variance (ANOVA) on body weight of Shikabrown breeder cocks pre-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	1.7436	0.3487	5.37	0.0001
Breed	1	0.9175	0.9175	14.14	0.0002
Week * Breed	5	0.1458	0.0292	0.45	0.8135

T-test on means of body weight for six weeks of Shikabrown cocks pre-infection

T-grouping	Mean	No. of cocks	Week
A	2.1140	50	3
A	2.0730	50	4
A	2.0580	50	5
A	2.0410	50	6
C	2.0172	50	2
C	1.8722	50	1

T-test for the means of the body weights of the strains of Shikabrown cocks pre infection.

T-grouping	Mean	No. of observations	Strain
A	2.0912	133	RSB
A	1.9794	167	WSB

Appendix 8: ANOVA on cloacal temperatures of Shikabrown breeder cocks pre-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	3.80866667	0.76173333	6.10	0.0001
Breed	1	0.32443998	0.32443998	2.60	0.1081
Week * Breed	5	1.04586136	0.20917227	1.67	0.1406

T-test on means of cloacal temperatures for six weeks on cloacal temperatures of Shikabrown cocks pre-infection

T-grouping	Mean	No. of cocks	Week
A	41.6812	50	2
A	41.6140	50	3
B	41.4760	50	5
C	41.4200	50	4
C	41.4160	50	1
C	41.3729	50	6

T-test on means of cloacal temperatures of strains of Shikabrown cocks pre-infection

T-grouping	Mean	No. of observations	Strain
A	41.5334	133	RSB
A	41.4672	167	WSB

Appendix 9: ANOVA on cloacal temperatures of Shikabrown cocks post infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	51.07429500	10.21485900	0.76	0.5780
TRT	1	9.38340833	8.38340833	0.63	0.4298
Breed	1	3.09681953	3.09681953	0.23	0.6312
Week * TRT	5	13.61136167	2.73827233	0.20	0.9605
Week * Breed	5	24.33845365	4.86769073	0.36	0.8736
TRT * Breed	1	42.52626224	42.52626226	3.17	0.0760

T-test on means of the cloacal temperatures of Shikabrown cocks

T-grouping	Mean	No. of cocks	Week
A	41.830	50	5
A	41.632	50	3
A	41.654	50	1
A	41.606	50	2
A	41.570	50	6
A	40.583	50	4

T-test for means of cloacal temperatures for control and infected Shikabrown cocks

T-grouping	Mean	No. of observations	TRT
A	41.655	150	Infected
A	41.320	150	Control

T-test for means of cloacal temperatures for strains of Shikabrown cocks

T-grouping	Mean	No. of observations	Strain
A	41.578	168	White
A	41.373	132	Red

Appendix 10: ANOVA on semen volume of Shikabrown breeder cocks pre-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	1.5643	0.313	4.55	0.0005
Breed	1	0.6192	0.619	9.01	0.0029
Week * Breed	5	0.2241	0.045	0.65	0.6601

T-test on means of semen volume of Shikabrown cocks pre-infection

T-grouping	Mean	No. of cocks	Week
A	0.5900	50	1
A	0.5420	50	5
A	0.5360	50	6
A	0.4400	50	2
A	0.4180	50	4
B	0.3960	50	3

T-test for the means of the semen volume of the strains pre-infection.

T-grouping	Mean	No. of observations	Strain
A	0.5275	167	RSB
A	0.4361	133	WSB

Appendix 11: ANOVA on spermatozoa motility of Shikabrown breeder cocks pre-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	5829.2400	1165.8480	2.61	0.0252
Breed	1	4522.4111	4522.4111	10.11	0.0016
Week * Breed	5	2441.1921	488.23427	1.09	0.3650

T-test on means of semen motility of Shikabrown cocks pre-infection

T-grouping	Mean	No. of cocks	Week
A	74.800	50	1
A	67.300	50	2
A	66.100	50	5
A	64.200	50	6
A	63.240	50	3
B	60.840	50	4

T-test on means of semen motility of strains of Shikabrown cocks

T-grouping	Mean	No. of observations	Strain
A	69.545	167	WSB
B	61.729	133	RSB

Appendix 12: ANOVA on semen concentration of Shikabrown breeder cocks pre-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	57.2099	11.4419	3.07	0.0102
Breed	1	9.2270	9.2270	2.48	0.1165
Week * Breed	5	4.2656	4.2656	1.15	0.3363

T-test on means of semen concentration of Shikabrown cocks pre-infection

T-grouping	Mean	No. of cocks	Week
A	3.702	50	6
A	3.430	50	5
A	3.352	50	1
A	2.888	50	2
A	2.696	50	3
B	2.466	50	4

T-test on means of semen concentration of strains of Shikabrown cocks pre-infection

T-grouping	Mean	No. of observations	Strain
A	3.246	167	WSB
B	2.892	133	RSB

Appendix 13: ANOVA on per cent live spermatozoa Shikabrown breeder cocks pre-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	5413.6667	1082.7333	2.20	0.0540
Breed	1	1851.7500	1851.7500	3.77	0.0532
Week * Breed	5	951.5753	190.31506	0.39	0.8574

T-test on means of per cent live spermatozoa of Shikabrown cocks pre-infection

T-grouping	Mean	No. of cocks	Week
A	78.100	50	1
A	74.100	50	5
C	70.300	50	2
C	69.300	50	4
C	68.700	50	3
C	64.700	50	6

T-test on means of per cent live spermatozoa of strains of Shikabrown cocks

T-grouping	Mean	No. of observations	Strain
A	73.084	167	WSB
A	68.083	133	RSB

Appendix 14: ANOVA on total sperm abnormalities of Shikabrown cocks pre-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	226.640	45.328	3.21	0.006
Breed	1	79.459	79.459	5.77	0.0169
Week * Breed	5	119.703	23.940	1.14	0.125

T-test on means of total spermatozoa abnormalities of Shikabrown cocks pre-infection

T-grouping	Mean	No. of cocks	Week
A	5.640	50	6
A	5.500	50	5
A	5.260	50	1
A	5.120	50	2
A	4.720	50	3
B	3.080	50	4

T-test on means of total spermatozoa abnormalities of strains of Shikabrown cocks pre-infection

T-grouping	Mean	No. of observations	Strain
A	5.359	167	WSB
B	4.323	133	RSB

Appendix 15: ANOVA on body weight of Shikabrown breeder cocks post-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	0.53759200	0.10757840	2.57	0.0269
TRT	1	0.75001200	0.75601200	18.10	0.0001
Breed	1	0.00587145	0.00597145	0.14	0.7070
Week * TRT	5	0.14966800	0.02993360	0.72	0.6113
Week * Breed	1	0.22683062	0.22683062	5.43	0.0205
Week*Breed	5	0.12167212	0.02433442	0.58	0.7133

T-test on means of body weight of Shikabrown cocks post infection

T-grouping	Mean	No. of cocks	Week
A	2.3080	50	6
A	2.2930	50	5
C	2.2250	50	4
C	2.2102	50	2
C	2.2080	50	3
C	2.2054	50	1

T-test on means of body weight of control and infected Shikabrown cocks post-infection

T-grouping	Mean	No. of observations	TRT
A	2.2918	150	Infected
A	2.1914	150	Control

T-test on means of body weight of strains of Shikabrown cocks post-infection

T-grouping	Mean	No. of observations	Strain
A	2.2466	132	WSB
A	2.2377	168	RSB

Appendix 16: ANOVA on semen volume of Shikabrown breeder cocks post infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	1.53866667	0.30773333	4.30	0.0001
TRT	1	0.17280000	0.17280000	2.41	0.1214
Breed	1	1.24156385	1.24156385	17.34	0.0001
Week * TRT	5	0.23920000	0.09784000	0.07	0.6480
Week * Breed	5	0.19237771	0.03847554	0.54	0.6480
Week*Breed	1	0.00051137	0.00051137	0.01	0.9327

T-test on means of semen volume of Shikabrown cocks post infection

T-grouping	Mean	No. of cocks	Week
A	0.4460	50	4
A	0.4300	50	2
A	0.4140	50	3
B	0.3960	50	5
B	0.3740	50	6
B	0.3560	50	1

T-test on means of semen volume of control and infected cocks post infection

T-grouping	Mean	No. of observations	TRT
A	0.3973	150	Infected
A	0.3493	150	Control

T-test on means of semen volume of strains of Shikabrown cocks post infection

T-grouping	Mean	No. of observations	Strain
A	0.4304	168	WSB
B	0.3008	132	RSB

Appendix 17: ANOVA on the semen colour of Shikabrown cocks post-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	13.7466667	2.74933333	2.38	0.0386
TRT	1	0.01333333	0.01333333	0.01	0.9145
Strain	1	14.51863636	14.51863636	12.59	0.0005
Week * TRT	5	6.46666667	1.29333333	1.12	0.3491
Week * Strain	5	3.15863636	0.63172727	0.55	0.7400
Week* Strain	1	2/95919659	2.95919659	2.57	0.1103

T-test on means of semen colour of Shikabrown cocks post-infection

T-grouping	Mean	No. of cocks	Week
A	2.160	50	6
A	2.020	50	4
A	1.860	50	5
A	1.820	50	2
A	1.740	50	3
B	1.480	50	1

T-test on means of semen colour of control and infected Shikabrown cocks post-infection

T-grouping	Mean	No. of observations	TRT
A	1.853	150	Infected
A	1.840	150	Control

T-test on means of semen colour of strain of Shikabrown cocks post-infection

T-grouping	Mean	No. of observations	Strain
A	2.042	168	WSB
B	1.598	132	RSB

Appendix 18: ANOVA on spermatozoa motility of Shikabrown breeder cocks post-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	4051.41667	810.28333	1.0	0.4206
TRT	1	7.363333	7.36333	0.01	0.9243
Strain	1	11727.67812	11727.67812	0.01	0.9243
Week * TRT	5	6163.41667	11727.67812	14.41	0.0002
Week * Strain	5	921.91296	184.38279	0.23	0.9508
Week* Strain	1	5713.26633	5713.26633	7.02	0.0005

T-test on means of spermatozoa motility of Shikabrown cocks post-infection

T-grouping	Mean	No. of cocks	Week
A	57.500	50	3
A	56.540	50	6
A	56.000	50	4
A	54.800	50	2
A	52.800	50	5
B	46.500	50	1

T-test on means of spermatozoa motility of control and infected Shikabrown cocks post-infection

T-grouping	Mean	No. of observations	TRT
A	54.180	150	Infected
A	53.867	150	Control

T-test on means of spermatozoa motility of strains of Shikabrown cocks post-infection

T-grouping	Mean	No. of observations	Strain
A	59.565	168	WSB
B	46.970	132	RSB

Appendix 19: ANOVA on spermatozoa concentration of Shikabrown cocks post-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	69.31936667	13.86387333	2.97	0.0124
TRT	1	2.10003333	2.10003333	0.45	0.5026
Strain	1	51.38001061	51.38001061	11.02	0.0010
Week * TRT	5	40.99456667	8.19891333	1.76	0.1213
Week * Strain	5	35.91995693	7.18399139	1.54	0.1770
TRT* Strain	1	8.78374086	8.78374086	1.88	0.1709

T-test on means of spermatozoa concentration of Shikabrown cocks post-infection

T-grouping	Mean	No. of cocks	Week
A	3.742	50	6
A	3.274	50	4
A	3.262	50	2
A	2.930	50	5
A	2.896	50	3
C	2.170	50	1

T-test on means of spermatozoa concentration of control and infected Shikabrown cocks post-infection

T-grouping	Mean	No. of observations	TRT
A	3.129	150	Infected
A	2.962	150	Control

T-test on means of spermatozoa concentration of strains of Shikabrown cocks

T-grouping	Mean	No. of observations	Breed
A	3.413	168	WSB
B	2.519	132	RSB

Appendix 20: ANOVA on per cent live spermatozoa concentration of Shikabrown cocks post-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	14061.41667	2812.28333	2.98	0.0123
TRT	1	1180.08333	1180.08333	1.25	0.2645
Strain	1	13750.9063	13750.90963	14.57	0.0002
Week * TRT	5	16475.41667	3295.08333	3.49	0.0045
Week * Strain	5	3063.22348	612.64480	0.65	0.6625
TRT* Strain	1	6886.24266	7886.24266	7.29	0.0073

T-test on means of per cent live spermatozoa of Shikabrown cocks

T-grouping	Mean	No. of cocks	Week
A	64.000	50	2
A	62.500	50	6
A	61.700	50	3
A	60.800	50	4
A	49.900	50	1
C	46.200	50	5

T-test on means of per cent live spermatozoa of control and infected Shikabrown cocks

T-grouping	Mean	No. of observations	TRT
A	59.500	150	Control
A	55.533	150	Infected

T-test on means of per cent live spermatozoa of strains of Shikabrown cocks

T-grouping	Mean	No. of observations	Breed
A	65.518	168	WSB
B	49.879	132	RSB

Appendix 21: ANOVA on total spermatozoa abnormalities of Shikabrown cocks post-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	684.5566667	136.9713333	2.64	0.0237
TRT	1	702.2700000	702.2700000	13.53	0.003
Strain	1	2.2722078	2.2722078	0.04	0.8344
Week * TRT	5	509.3900000	101.8780000	1.96	0.0842
Week * Strain	5	756.9755844	151.3951169	2.92	0.0138
TRT* Strain	1	158.1849433	158.1849433	3.05	0.0819

T-test on means of total spermatozoa concentration of Shikabrown cocks post-infection

T-grouping	Mean	No. of cocks	Week
A	8.760	50	4
A	6.380	50	2
A	6.140	50	3
B	5.080	50	5
B	4.660	50	6
B	4.160	50	1

T-test on means of spermatozoa concentration of Shikabrown cocks post-infection

T-grouping	Mean	No. of observations	TRT
A	7.393	150	Infected
B	4.333	150	Control

Appendix 22: ANOVA on cloacal temperatures of Shikabrown cocks post-infection

Source	DF	ANOVA SS	Mean Square	F-value	PR>F
Week	5	51.07429500	10.21485900	0.76	0.5780
TRT	1	9.38340833	8.38340833	0.63	0.4298
Breed	1	3.09681953	3.09681953	0.23	0.6312
Week * TRT	5	13.69136167	2.73827233	0.20	0.9605
Week * Breed	5	24.33845365	4.86769073	0.36	0.8736
TRT*Breed	1	42.52626224	42.52626224	3.17	0.0760

T-test on the means of the cloacal temperatures of Shikabrown cocks post-infection

T-grouping	Mean	No. of cocks	Week
A	41.830	50	5
A	41.682	50	3
A	41.654	50	1
A	41.606	50	2
A	41.570	50	6
A	40.583	50	4

T-test on the means of the cloacal temperatures of control and infected Shikabrown cocks

T-grouping	Mean	No. of observations	TRT
A	41.655	150	Infected
A	41.320	150	Control

T-test on the means of the cloacal temperatures of the strains of Shikabrown cocks

T-grouping	Mean	No. of observations	Strain
A	41.578	168	WSB
A	41.373	132	RSB

Appendix 23: Correction analysis of semen characteristics of Shikabrown cocks 1 – 6 weeks pre-infection

	BWT	VOL	COL	MOT	CONC.	PC LIV
BWT	0.00	0.0005	0.7682	0.0188	0.8353	0.3815
VOL	0.0005	0.0	0.0001	0.0001	0.0001	0.0001
COL	0.7682	0.0001	0.0	0.0001	0.0001	0.0001
MOT	0.0188	0.0001	0.0001	0.0	0.0001	0.0001
CONC	0.8353	0.0001	0.0001	0.0001	0.0	0.0001
PC LIV	0.3815	0.0001	0.0001	0.0001	0.0001	0.0001
PC ABN	0.1281	0.0012	0.0067	0.0001	0.0283	0.0001

($P<0.05$)

Appendix 24: Correction analysis of semen characteristics of Shikabrown cocks 1 – 6 weeks post infection

	BWT	VOL	COL	MOT	CONC.	PC LIV
BWT	0.00	0.4009	0.0473	0.3391	0.4097	0.1618
VOL	0.4009	0.0	0.0001	0.0001	0.0001	0.0001
COL	0.0473	0.0001	0.0	0.0001	0.0001	0.0001
MOT	0.3341	0.0001	0.0001	0.0	0.0001	0.0001
CONC	0.4097	0.0001	0.0001	0.0001	0.0	0.0001
PC LIV	0.1618	0.0001	0.0001	0.0001	0.0001	0.0001
PC ABN	0.7478	0.0001	0.0001	0.0001	0.0001	0.0001

($P<0.05$)

Appendix 25: Hemagglutination inhibition Newcastle disease antibody titres of infected and control Shikabrown cocks (A – Infected; B = Control)

Group	Weeks post infection						Group	Weeks post infection					
A	**1**	**2**	**3**	**4**	**5**	**6**	**B**	**1**	**2**	**3**	**4**	**5**	**6**
014	6	9	8	9	8	6	**048**	9	7	7	8	7	4
001	10	9	9	6	6	4	**045**	5	2	4	3	4	2
019	7	10	9	7	7	6	**022**	7	2	3	3	4	1
016	5	10	10	10	8	7	**008**	5	5	4	3	4	2
032	6	10	6	8	8	5	**018**	4	10	4	4	3	2
002	8	10	9	9	8	6	**028**	5	5	4	3	4	2
010	6	8	8	7	7	5	**026**	4	5	2	3	3	2
024	10	6	7	7	5	4	**039**	3	5	2	3	3	2
030	8	9	7	8	8	5	**006**	3	7	4	3	4	1
046	10	9	7	8	7	5	**013**	4	4	3	3	4	2
007	5	10	9	8	7	5	**023**	2	3	2	2	2	-
015	10	8	6	9	7	5	**041**	3	4	3	3	3	2
036	9	10	10	9	8	6	**003**	1	3	1	1	1	-
035	5	10	8	9	7	5	**044**	7	3	3	3	3	1
042	6	10	10	10	8	5	**089**	4	6	2	4	4	2
047	10	10	8	10	8	7	**040**	4	1	0	1	-	-
011	10	10	6	10	8	7	**021**	3	6	2	3	2	3
050	7	10	10	9	8	6	**033**	4	3	2	1	3	0
009	9	10	8	10	9	7	**049**	4	2	2	2	-	0
025	7	10	9	7	6	4	**034**	2	2	0	-	1	1
027	4	8	8	7	7	6	**020**	4	1	1	-	0	-
031	10	9	6	6	5	4	**038**	2	2	3	1	-	-
012	10	10	8	8	6	5	**037**	4	3	4	2	3	2
004	8	10	8	10	9	6	**045**	5	5	5	3	4	2
017	5	10	7	6	7	5	**043**	7	4	2	3	4	0