THE EFFECT OF DIETARY CRUDE PROTEIN, ORGANIC SELENIUM AND VITAMIN E ON FERTILITY AND SEMEN QUALITY OF BROILER BREEDER MALES

by

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As the candidate's supervisor I have/have not approved this dissertation for submission.

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ABSTRACT

There are negative influences of selection for broiler growth on the reproductive ability of broiler breeder parents. This is mostly due to problems related to excessive body weight, such as an inability to achieve successful cloacal contact during natural mating. There is also an age-related decline in fertility of broiler breeders. In attempts to prolong the fertile period of the breeders, various forms of management techniques have been employed. These include tools such as feed restriction, photoperiod management, spiking, and possibly even using artificial insemination.

The first objective of this thesis was to investigate the possible benefits of feeding broiler breeder males diets containing lower crude protein levels, than given to the females, as a means of possibly tempering growth rate or improving semen quality and fertility. There was a definite improvement in the ability of the spermatozoa of the males on a lower crude protein diet to survive in the female reproductive tract. Males that received higher levels of crude protein were at a disadvantage in that fewer males yielded semen in response to abdominal massage.

The second objective of this thesis was to assess the possible benefits in semen quality and fertility, when supplementing the male diets with additional vitamin E or Se in the form of Sel-Plex®. No significant effect of treatment was observed on egg fertility or semen quality.

PREFACE AND DECLARATIONS

The experimental work described in this dissertation was carried out in the School of Agricultural Sciences and Agribusiness, University of KwaZulu-Natal, Pietermaritzburg, from January 2005 to December 2008, under the supervision of Mrs Nicola C. Tyler.

I, …… declare that

1. The research reported in this dissertation, except where otherwise indicated, is my original research.

2. This dissertation has not been submitted for any degree or examination at any other university.

3. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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ABBREVIATIONS USED IN THE TEXT

- AI - artificial insemination
- ANOVA - analysis of variance
- ATP - adenosine triphosphate
- BA - predicted fertility status of an egg according to Brillard & Antoine (1990)
- BW - body weight
- CP - crude protein
- DAPI - diamidinophenylindole
- *dw* - recessive sex-linked dwarf gene
- *Dw* - dominant normal growth gene
- ED - limited every day feeding programme
- FSH - follicle stimulating hormone
- GnRH - gonadotrophin releasing hormone
- GSH-Px - glutathione peroxidise
- IPVL - inner perivitelline layer
- LH - luteinizing hormone
- LSD - least significant difference
- ME - metabolizable energy
- OPVL - outer perivitelline layer
- $OPVL_{sperm}$ - sperm embedded within the OPVL
- P - probability
- PBS - phosphate buffered saline
- PUFA - polyunsaturated fatty acids
- PVL - perivitelline layer
- SAD - skip-a-day feeding programme
- Se - selenium
- S.E. - standard error of observations
- s.e.m. - standard error of mean
- SQI - sperm quality index
- SST - sperm storage tubules
- W - predicted fertility status of an egg according to Wishart (1997)

CHAPTER 1

GENERAL INTRODUCTION

Through intense genetic selection and improved nutritional management there has been a rapid increase in the growth rate of broiler breeders. The modern broilers therefore have an improved feed conversion and decreased age to slaughter (Havenstein *et al.*, 2003). Broiler breeders are required to pass the genes for these traits to as many offspring as possible. Therefore, the objectives in commercial broiler breeder operations are to obtain a maximum number of eggs during the production cycle, while still achieving the correct egg size to obtain day old chicks of a "commercially acceptable" body weight (Brillard, 2001).

Intense selection for growth rate and efficiency in broilers has, however, altered the birds' ability to regulate feed intake (Bokkers & Koene, 2003). This has created a situation, where under *ad libitum* feeding, broilers can be overfed, since they will consume food at near gut capacity (Nir *et al.*, 1978). This over-consumption of food is manifested in increased carcass fatness (Emmerson, 1997) and reduced fecundity (Siegel & Dunnington, 1985). Very heavy males also experience a higher incidence of foot and leg disorders which interfere with locomotion as well as natural mating (Hocking & Duff, 1989).

The broiler industry has, therefore, been forced to employ a number of management schemes in order to maintain relatively high flock fertility. These include selection for fertility traits, photoperiod management and feed restriction. Breeder companies therefore recommend strain-specific procedures for nutrient allocation and photoperiod management.

Chapter 2 is a review of the factors affecting the reproductive soundness of broiler breeder males. Particular attention was given to the management of reproduction through the manipulation of feeding regimes. Chapter 3 is a summary of a preliminary study that was done in order to perfect some experimental techniques and to investigate whether there is any effect of dietary crude protein levels on semen quality of breeder males. In Chapter 4 the reproductive performance of broiler breeder males fed different dietary levels of crude protein was compared. Chapter 5 evaluates the use of feed supplementation, in the form of organic selenium and vitamin E, on broiler breeder male semen quality and fertility.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Reproductive traits and body weight (or growth rates) of broilers are negatively correlated (Soller & Rappaport, 1971, Siegel & Dunnington, 1985, Lake, 1989). High levels of fertility (above 95 percent) can be attained during the early breeding period (30 to 49 weeks of age), but fertility rapidly declines after 40 to 45 weeks of age (Casanovas, 2002). It is, therefore, important to manage the parent stock in such a manner so as to maintain a relatively high flock fertility and to try to suppress the age-related decline in fertility.

The following review will consider factors affecting the reproductive physiology and fertility of broiler breeder males in particular. Some measures of semen quality and fertility are discussed. The age-related decline in fertility of breeders and some management factors that have been implemented in an attempt to sustain fertility were also reviewed, focussing particularly on feeding practice.

2.2 MALE REPRODUCTIVE PHYSIOLOGY

2.2.1 The Gonads and Associated Sexual Organs

The paired testes of the male fowl are situated within the abdomen and are attached by ligaments to the dorsal body wall on either side of the midline, adjacent to the anterior parts of the kidneys and just posterior to the lungs (Lake & Stewart, 1978). They have a dual function: spermatogenic and endocrine. Both testes are functional in the male, whereas, in the female, only the left ovary is functional (Etches, 1996). Puberty in the cockerel is defined as the age of first appearance of spermatozoa in ejaculated semen and occurs between 16 and 24 weeks of age (Howarth, 1995). The initial semen quality is relatively inferior, so sexual maturity has also been defined as the time when testis growth reaches a maximum and the number and quality of spermatozoa is maximal (de Reviers & Williams, 1984).

Gonadal size in the fowl is influenced by age, sex, day-length and physiological condition of the bird (Bennett, 1947). The weight of the paired testes increases from 2-4 g to 25-35 g, as sexual maturity is attained (Etches, 1996). There is a direct relationship between sperm production and testis size (Ansah *et al.*, 1985, Lee *et al.*, 1999); the latter usually correlates positively with body size during breeding (Wilson *et al.*, 1988, Fontana *et al.*, 1990, Leeson & Summers, 2000). The expression of a sexual ornament, the comb, reflects testicular mass and thus the rate of sperm production in male fowl (Pizzari *et al.*, 2004). Testicular size can also be determined by ultrasound images in adult roosters with little physical damage to the intestine or cloaca (Richardson *et al.*, 2002).

Sperm production occurs at a fairly constant rate of about 100 million sperm/g of testes weight per day; this is not affected by mating or semen collection frequency (Leeson & Summers, 2000). The rate of sperm passage through the excurrent ducts as well as the quality and fertilising ability of the spermatozoa are, however, affected by the frequency of semen collection (de Reviers, 1973). Brown and McCartney (1986) reported that testes weights of over 7 g were required for the production of collectable semen, while Hocking (1990) observed a high proportion of infertile males when testes weights were below 11 g. The testes ability to produce spermatozoa is also closely linked to Sertoli cell number in the testes; the proliferation of which occurs before the onset of sexual maturity (de Reviers & Williams, 1984, Etches, 1996).

Spermatogenesis in the fowl proceeds at the internal body temperature of close to 41 ^{o}C (Leeson & Summers, 2000) and newly formed spermatozoa may appear in the ejaculate after only 13 to 15 days (Howarth, 1995). The ducts that convey spermatozoa away from the testis are the efferent ducts, connecting ducts, epididymis, and the ductus deferens or vas deferens (Howarth, 1995). The caudal end of each vas deferens opens into the cloaca via a papilla that is located in close proximity to a rudimentary phallus (Howarth, 1995). Semen is stored in the lower portion of the vas deferens (Lake & Stewart, 1978) and is expelled from this portion into the cloaca at ejaculation (Etches, 1996). During sexual excitation, the lateral phallic folds of the ventral cloaca become engorged with lymphatic fluid and protrude, forming a depression through which the semen flow is directed (Etches, 1996).

2.2.2 Hormonal Profiles and Secondary Sexual Characteristics

The endocrine functioning of the testes are regulated by the secretion of gonadotrophins from the anterior pituitary gland and gonadotrophin releasing hormone (GnRH) from the hypothalamus (Etches, 1996). The two major gonadotropic hormones that are involved in testicular regulation are luteinizing hormone (LH) and follicle stimulating hormone (FSH). Luteinizing hormone stimulates the production of androgens from the Leydig cells of the testes (Etches, 1996). Several androgens are produced by these cells, the major hormone in blood being testosterone (Appleby *et al.*, 2004). As the body weight of the male increases, the concentration of LH increases, and maximum concentrations of LH occur after sexual maturity is attained (Etches, 1996). The Sertoli cells of the testes are under the control of FSH and testosterone. Testosterone enters the Sertoli cells and combines with an androgen-binding protein (which is FSH dependent), securing a local concentration of testosterone close to the spermatocytes (Howarth, 1995). This stimulates spermatogenesis and further germ cell development (Howarth, 1995).

In the sexually mature male, the plasma concentration of LH is maintained by a negative feedback loop: high concentrations of testosterone inhibit the output from the hypothalamus of GnRH which controls LH (Appleby *et al.*, 2004). As the LH level falls, so does the production of testosterone, and this in turn ensures that GnRH is again produced, to release more LH and thus raise the androgen output once more (Etches, 1996). Another hormone that is involved in a negative feedback loop is inhibin. It is thought to be produced by the Sertoli cells and feeds back on the anterior pituitary to decrease the secretion of FSH (Howarth, 1995).

At each stage of sexual maturation, hormone production depends on the interaction between the hypothalamus, the pituitary gland and the gonadal steroids, and these interactions change during maturation (Etches, 1996). The increase in plasma concentrations of LH associated with sexual maturation in the male is the consequence of a gradual loss in the ability of the androgens to suppress the secretion of LH from the pituitary gland (Etches, 1996). Androgen production coincides with the development of spermatogenesis, testicular growth and the development of secondary sexual characteristics, such as comb growth and crowing. Increased testosterone secretion is also

associated with the expression of aggressive behaviour during territorial encounters with rivals, and is responsible for mating behaviour, especially libido (Howarth, 1995).

2.2.3 Semen Characteristics

Semen consists of spermatozoa and seminal plasma. The latter is derived from the testes and the excurrent ducts (Etches, 1996). At the time of ejaculation a transparent lymph-like fluid of cloacal origin may also be added to the semen in varying amounts (Fujihara, 1992). It has been suggested that this fluid is involved in activating the motility of the spermatozoa (Howarth, 1995). Good quality semen with high sperm density usually appears pearly-white and thick, while semen of low density appears greyish or watery (Lake & Stewart, 1978). Any observed discolouration of semen may be indicative of poor quality. Semen used for artificial insemination (AI) should have no solid white, chalky deposits (indicating urine contamination), dark masses (indicating faeces) or brownish red pigments (indicating the presence of erythrocytes), as these contaminants depress the fertilising capacity of semen to varying extents (Etches, 1996).

2.2.4 Semen Evaluation

The quality of spermatozoa is of prime importance with respect to their survival *in vivo* or *in vitro*, their fertilising ability, and to the vitality of the embryos (de Reviers & Williams, 1984). The following are methods used in an attempt to quantify semen quality.

2.2.4.1 Semen volume and concentration

According to Wilson *et al.* (1979), the individual effects of concentration and volume upon fertility of broiler breeder males are interdependent upon one another. It is essential to know sperm concentrations in order to calculate the appropriate levels of dilution for AI (see Section 2.4.2). It may also assist in ranking individual males with regards to their potential fertility when using AI. On average, semen of the fowl contains between three and eight billion spermatozoa per ml (Lake & Wishart, 1984, Donoghue & Wishart, 2000). The lowest concentrations may be indicative of a gross contamination with transparent lymphatic fluid (Lake & Stewart, 1978).

The concentration of spermatozoa in a semen sample can be determined by either direct counting of a diluted sample with a haemocytometer or by indirect methods, which are generally less time consuming and more reliable (Brillard & McDaniel, 1985, Leeson & Summers, 2000). The two commonly used indirect methods are packed cell volume, using a haematocrit, and optical density, using a spectrophotometer (Howarth, 1995). In order to assess the volume of ejaculates from individual males, the semen can be weighed, as the density of semen is assumed to be 1 mg μl^{-1} (Etches, 1996).

2.2.4.2 Spermatozoa viability (percentage of live sperm)

The percent dead spermatozoa in a semen sample has been found, in a review by Wilson *et al.* (1979), to be negatively correlated with fertility. A significant ($P<0.05$) decrease in fertility has been reported when the percent dead spermatozoa was greater than one percent (Wilson *et al.*, 1969). A trypan blue stain was used in this study to determine live and dead spermatozoa in cock semen. Dead cells were distinguished by the head being wholly or partially stained blue, while live cells were unstained. Sperm viability can also be determined by a nigrosin-eosin stain (Leeson & Summers, 2000). Live viable sperm do not take up the pink-coloured eosin stain, and so remain white, on the blue (nigrosin) background, while dead sperm take up the eosin, and appear pink. When viewed under a microscope at 80 to 100 times magnification, a field of view could therefore be measured for live versus dead sperm (Leeson & Summers, 2000).

2.2.4.3 Spermatozoa motility, mobility and metabolic activity

An objective colourimetric technique for estimating the capacity of fowl spermatozoa to reduce a tetrazolium dye (colourless) to red formazan pigment has been described (Chaudhuri & Wishart, 1988). This assay was found to be a measure of the metabolic activity of fowl sperm, and the rate of dye reduction was shown to be correlated with oxygen utilisation by the spermatozoa. However, this test does not differentiate between a sample of poorer quality with a high concentration of sperm, and a sample of better quality with a lower concentration of sperm (Chaudhuri & Wishart, 1988). In addition, even though this assay was found to be strongly correlated with sperm motility, morphology, ATP content and fertilising ability, it appeared less correlated with fertilising ability than the other variables (Chaudhuri *et al.*, 1988).

Another test that measures the relative metabolic activity of semen is the methylene blue reduction test. In this test, the rate at which semen, diluted with a yolk-citrate diluent, reduces a solution of methylene blue is determined. Methylene blue looses its deep blue colour when reduced by the addition of two atoms of hydrogen, thus the colour of the solution changes from green to yellow, as the sperm cells release hydrogen ions as they metabolise (Beck & Salisbury, 1943). This test is largely dependent upon the concentration and motility of the spermatozoa (Beck & Salisbury, 1943) and on the number of live spermatozoa in a semen sample (Selvan, 2007).

The motility of poultry sperm has been measured objectively by spectrophotometry, video microscopy and digital image analysis (Froman & McLean, 1996). Sperm motion has also been measured by using a method involving the sperm penetration of Accudenz® at body temperature (Froman & McLean, 1996). Accudenz® is a non-ionic, biologically inert cell separation medium and when a sperm suspension is overlaid on this medium, a distinct interface forms because of the difference in density between the two media (Froman & Feltmann, 2000). After a short incubation interval, the absorbance of the Accudenz® solution is determined with a photometer (Froman & McLean, 1996) and this is proportional to the number of sperm that have entered the Accudenz® layer (Froman & Feltmann, 2000). It was found that this assay does not give information about individual sperm, and that it is an assessment of the mobility of a sperm population (Froman & McLean, 1996). The authors concluded that it is sperm mobility, rather than sperm motility *per se* that enables sperm sequestration within the hen's sperm storage tubules.

Unlike motility assessment, in which the percentage of moving sperm is estimated, sperm mobility is a measure of the proportion of sperm in a semen sample with a powerful and relatively linear forward motion (Donoghue, 1999). It can be explained in terms of the concentration of motile sperm and the straight-line velocity of individual sperm cells (Froman & Feltmann, 2000), and, as such, is expected to give a better idea of fertilising ability than a motility assessment. The hatchability of eggs laid by hens inseminated with sperm from a high mobility phenotype was found to be 10 percent greater than that of hens inseminated with sperm from an average phenotype (Froman *et al.*, 1997). The mobility of sperm has also been found to have an influence on the rate at which sperm are lost from the female oviduct (Froman *et al.*, 2002). High mobility sperm were lost at a significantly slower rate and, therefore, retained their fertilising capacity for longer. Sperm mobility has

been shown to be a quantitative (Froman & Feltmann, 1998), heritable (Froman *et al.*, 2002) trait affecting male reproductive fitness in chickens (Froman *et al.*, 1999). Sperm mobility was also found to be correlated with sperm ATP content, but does not appear to be correlated with body weight or testes weight (Froman & Feltmann, 1998, Bowling *et al.*, 2003). Thus, male reproductive efficiency in breeder flocks could potentially be improved, by selecting for the sperm mobility trait, without compromising body weight (Froman, 2006). The commercial sperm mobility assay has been modified to exclude glucose from all of the media (Froman, 2006).

Sperm motion can also be assessed by an instrument called the Sperm Quality Analyzer® (McDaniel *et al.*, 1997b) that quantifies the amplitude and frequency of disruption of a light beam by sperm within a capillary tube (Parker *et al.*, 2000). It has been claimed that this assay provides an estimate of the overall quality of sperm from broiler breeder males by reflecting sperm concentration, viability, and motility in a single value, the sperm quality index (SQI) (McDaniel *et al.*, 1998). The SQI has been shown to be positively correlated ($r = 0.73$) with rooster fertility (Parker *et al.*, 2000).

A study was conducted to test the capacity of the Sperm Quality Analyzer® to predict sperm mobility phenotype and it was found that the SQI does not differentiate among sperm mobility phenotypes (Froman *et al.*, 2003). It was suggested that only the sperm mobility assay affords conditions in which the consequences of variation in average straight line velocity at body temperature become evident over time (Froman *et al.*, 2003). However, the accuracy of the SQI assay has been shown to be sensitive to the semen dilution rate (Parker & McDaniel, 2003). A 10-fold dilution was found to be the best dilution for the SQI to predict fertility and determine semen quality; dilution beyond this point appeared to alter semen quality, making the SQI less accurate (Parker & McDaniel, 2003). It appears that sperm motility, ATP utilization, gas exchange, and ionic balance are altered by diluent type and rate of dilution and that these alterations in semen quality are exacerbated by higher semen dilutions (Parker & McDaniel, 2006). In the study conducted by Froman *et al*. (2003), semen was diluted up to 40-fold before SQI analysis, possibly affecting the accuracy of their findings; thus another study was done to re-examined these two methods of assessing sperm motility, using a 10-fold dilution for the SQI (Dumpala *et al.*, 2006). It was concluded that both the SQI and sperm mobility assay are indicative of sperm motility (Dumpala *et al.*, 2006).

2.2.4.4 Spermatozoa morphology

The sperm cells of poultry are long, cylindrical, and tapered at both ends (Etches, 1996). Light microscopy is used to evaluate spermatozoa gross morphology; this may provide information on testicular function and also aid in predicting the fertility potential of a male (Howarth, 1995). Males with large numbers of abnormal spermatozoa generally have poor fertility, due to failure of such spermatozoa to reach the utero-vaginal sperm storage tubules in the female (Ogasawara *et al.*, 1966). Morphological abnormalities can occur in the acrosome, head, mid-piece or tail regions of poultry spermatozoa (Lake & Stewart, 1978, Alkan *et al.*, 2002). Turkey and cock semen have similar types of morphological defects and the most frequent defects are observed at the acrosome and mid-piece, suggesting that these areas are most susceptible to environmental factors (Alkan *et al.*, 2002).

From the above section, it is evident that a number of techniques have been used in attempts to quantify semen quality of broiler breeder males. It has, however, been suggested that more than one semen characteristic needs to be assessed, in order to predict fertility with greater accuracy (Wilson *et al.*, 1979).

2.3 SPERM STORAGE AND FERTILITY

Successful fertilisation in the fowl depends on the completion of a set of steps as follows: sperm deposition at the distal end of the female tract; ascent into the tract; sperm storage in the hen; sperm movement up the tract to the site of fertilisation; sperm binding to the outer membrane of the ovum; acrosome reaction; fertilisation; and development to yield progeny (Barbato *et al.*, 1998). In the following section, some of the factors involved with sperm transport, selection and storage, as well as some techniques that have been used to assess fertility of oviposited eggs, will be discussed.

2.3.1 Sperm Transport, Selection and Storage in Oviduct

When a hen is inseminated, oviposition of fertile eggs will occur over a period of time without the need for further AI or copulation. This period is known as the fertile period and is brought about by storage of spermatozoa in the sperm storage tubules (SST) in the

oviduct of the hen (Bakst *et al.*, 1994). The length of the fertile period is characteristic of each avian species and is slightly longer than the time taken for a hen to lay a clutch, so that all eggs laid have the opportunity to be fertilised (Wishart & Staines, 1999). Environmental regulation and genetic selection in poultry have enabled hens to lay continuously over prolonged periods, however, their fertile period has not increased equivalently and thus, poultry hens will lay unfertilised eggs unless they are inseminated periodically (Wishart & Staines, 1999). Fertility in chickens begins to decline six to seven days after insemination (Etches, 1995).

Sperm storage regions are situated at the uterovaginal junction, the distal infundibulum and the proximal magnum; the uterovaginal junction being the primary sperm storage site in the hen (Bobr *et al.*, 1964, Bakst *et al.*, 1994). Significant ($P<0.05$) differences have been found between a broiler strain and an egg-laying strain of chickens in terms of the numbers of SST (Van Krey *et al.*, 1971). The numbers of SST were significantly lower in the broiler strain hens and layer type hens had a significantly higher percentage of spermatozoa stored in the SST. The breed of the males also appears to have an effect on the ability of the inseminated sperm to survive in the hens as semen from meat-strain males resulted in a significantly ($P<0.01$) lower duration of fertility than semen from Leghorn-strain males (Taneja & Gowe, 1961, 1962).

Spermatozoa are packed into the lumen of the SST (Figure 2.1) in bundles of 100 to 200 cells (Etches, 1995) and are orientated with their long axis parallel to that of the gland and with their heads pointed towards the blind distal end of the SST (Tingari & Lake, 1973). Only metabolically active spermatozoa with intact plasma membranes are stored in the SST (Bakst *et al.*, 1994). Each population of stored sperm represents between 0.22 (McDaniel *et al.*, 1997a) and 0.9 (Bakst *et al.*, 1994) percent of the sperm number inseminated. McDaniel *et al*. (1997b) showed that insemination of hens with 25, 50 or 100 million spermatozoa resulted in a linear increase in the number of spermatozoa stored in the uterovaginal junction; but that insemination of hens with 328 million spermatozoa produced no increase in sperm storage over insemination with 100 million spermatozoa. Sperm from two different inseminations generally segregate into different storage tubules and thus the mechanism of last-male precedence does not appear to be due to the stratification of sperm within the SST of fowl (King *et al.*, 2002).

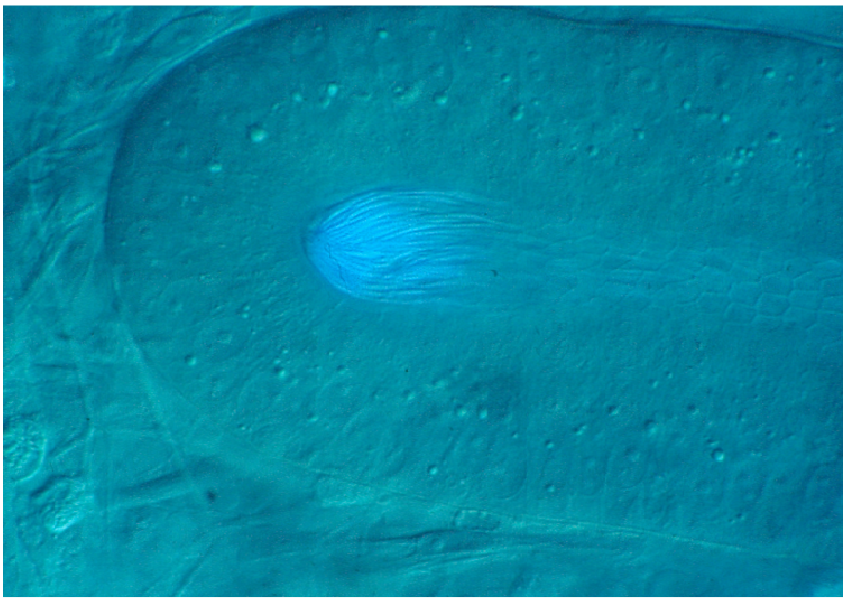

Figure 2.1 *Spermatozoa stored in the lumen of the SST of a hen (Brillard, J.P. - no published reference).*

Sperm are slowly and continuously released from the SST and are transported to the infundibulum where they may populate the infundibular SST or continue through to the ostium of the infundibulum into the abdominal cavity (Bakst *et al.*, 1994). Sperm must overcome a number of obstacles in order to reach the site of fertilisation. These include contractions of the oviduct (responsible for the transport of the egg mass towards the vagina) and secretion of albumen and the other structural elements forming the shell membranes and shell (Bakst *et al.*, 1994). Stored sperm are released from the infundibular SST (perhaps due to the distension of the infundibular mucosa when an ovum passes into the infundibulum) and become associated with the ovum (Bakst *et al.*, 1994).

Taneja and Gowe (1961, 1962) investigated the effect of semen dosage by artificial insemination on the number of 'skipped infertile eggs' (defined as the number of infertile eggs laid between the second day after insemination and the day the last fertile egg was laid). The mean number of skipped infertile eggs per bird was significantly ($P<0.01$) higher for small doses than for larger doses (Taneja & Gowe, 1962). It was suggested that this could be related to the probability of an ovum being fertilised when passing through the infundibulum, and that this would be determined to some extent by the density of spermatozoa in that area of the oviduct (Taneja & Gowe, 1962).

Fertilisation is influenced by the number of sperm inseminated (Burrows & Quinn, 1938, Munro, 1938, Taneja & Gowe, 1961, 1962). Taneja and Gowe (1962) observed that fertility gradually increased with the amount of semen inseminated till a plateau was reached (at 0.01 ml of undiluted semen) of where a further increase in the volume of semen inseminated did not result in a significant rise in fertility. There is also a relationship between the size of the insemination dose and the duration of fertility; the duration increases as the dosage of spermatozoa is increased (Taneja & Gowe, 1961, 1962, Brillard *et al.*, 1989). Brillard and McDaniel (1986) found that doses as high as 250 million spermatozoa (125 million spermatozoa administered on two consecutive days) do not saturate the SST in young or old hens. Higher doses (250 million spermatozoa inseminated on two consecutive days) were found to sustain fertility during the second week post-insemination (Brillard & McDaniel, 1986). Brillard and McDaniel (1986) suggested that changes in doses for AI act more to modify the duration, than maximum levels, of fertility.

2.3.2 Evaluation of Sperm: Egg Interactions

While previous methods mentioned are used to analyse semen quality, it is important to look at the fertilising ability of the spermatozoa, and their interaction with the egg. The following methods can be used to visualise and estimate the numbers of spermatozoa that associate with the egg during the time of fertilisation. These methods are also indicative of the fertilising potential of the sperm.

2.3.2.1 Sperm holes in perivitelline membrane

Sperm encounter the ovum in the infundibulum within approximately 15 minutes of ovulation (Wishart & Staines, 1999). After binding to the outer proteinaceous layer of the ovum, the inner perivitelline layer (IPVL), the spermatozoa are triggered to undergo an acrosome reaction, releasing acrosomal proteases (Wishart *et al.*, 2001). These enzymes hydrolyse a small hole of about 10 to 18 μm in the IPVL, through which a single spermatozoon will enter to fertilise the ovum (Koyanagi *et al.*, 1988).

The sperm-perivitelline interaction can be determined either *in vitro* (Bramwell & Howarth, 1992) or *in vivo* (Bramwell *et al.*, 1995). The technique used by Bramwell *et al.* (1995) was developed to assess the number of cock spermatozoa penetrating the

perivitelline layer (PVL) in oviposited eggs *in vivo*. It is a relatively simple technique that does not need any specialised equipment. The only requirements are a weak sodium chloride solution, 20 percent formalin, several drops of Periodic Acid Schiff's reagent and a light microscope. Staining with the Schiff's reagent makes the distinct holes in the sample of the PVL visible under a light microscope. These holes, which are assumed to be sites of spermatozoa penetration, are counted at 100 times magnification.

Bramwell and Howarth (1992) incubated cock spermatozoa with PVL from recently ovulated ova of the hen *in vitro*. A correlation was found between sperm concentration and the number of spermatozoa attaching to the PVL and undergoing an acrosome reaction. A preferential attachment of the spermatozoa to the PVL directly over the germinal disc of the hen's ovum has been found (Bramwell *et al.*, 1995, Wishart, 1997). Bramwell *et al.* (1995) reported that a mean number of three holes in the inner PVL from over the germinal disc region coincided with a mean fertility of 75 percent. Bramwell *et al.* (1995) also found a positive correlation between sperm penetration of the germinal disc PVL and fertility of eggs laid by hens after either natural mating or AI.

2.3.2.2 Sperm trapped in oviposited eggs

The interaction of spermatozoa with the IPVL is arrested following the secretion of the outer perivitelline layer (OPVL) by the cells of the distal infundibulum (Wishart *et al.*, 2001). Spermatozoa that are in the vicinity of the ovum, but have not penetrated the IPVL, become trapped in the protein fibrils of the forming OPVL (Wishart & Staines, 1999, Wishart *et al.*, 2001).

It has been shown that there is a significant positive correlation between the number of sperm passively trapped in the PVL of the oviducal egg and the number of sperm residing in the sperm storage tubules (Brillard & Antoine, 1990). It was suggested that the sperm-storage capacity of the oviduct and the quality of the semen sample could be estimated on the basis of the numbers of spermatozoa embedded in the PVL of a laid egg (Brillard & Antoine, 1990, Wishart, 1997). The number of spermatozoa interacting with the PVL has been found to be related to the insemination dose, the number of oviducal sperm, and the probability of fertilisation (Wishart & Staines, 1999). This was found to be the case, not just for one egg, but for subsequent eggs laid by the same hen.

2.4 ARTIFICIAL INSEMINATION

2.4.1 Semen Collection

A non-invasive method of semen collection from roosters, the "abdominal massage method" (Burrows & Quinn, 1937), is the technique that is still used to date. The reasons why simple abdominal massage stimulates ejaculation of semen by roosters are not apparent, as this technique does not resemble natural mating (Etches, 1996). One person would usually hold the male, restraining the wings while the legs are firmly held. Another person would then stimulate the male by firmly, but gently, massaging the abdominal region around the pelvic area with one hand, while simultaneously stroking the back of the bird behind the wings towards the tail with firm rapid strokes with the other hand (Bakst & Brillard, 1995). This causes an erection of the phallus, at which time the handler gently squeezes the cloaca, expressing semen through the external papillae of the vas deferens and into a collection container. A repeat massage may yield extra spermatozoa but will invariably also increase the contamination of the semen with a large volume of watery fluid, reducing the number of spermatozoa contained in a unit volume (Lake & Stewart, 1978). The male should be picked up quickly but gently and manipulated immediately in order to obtain the best response (Lake & Stewart, 1978). It is very important that the semen collector exercises the responsibility of inspecting and, if necessary, rejecting a poor quality semen sample, especially if the semen from more than one male is pooled (Bakst & Brillard, 1995).

It is difficult to collect semen from young birds or males that do not have any prior experience of semen collection (Lee *et al.*, 1999); thus it is very important to train the birds in order to get a positive response to the abdominal massage method. To obtain the maximum number of spermatozoa semen should be collected frequently. Semen collection increases the rate of sperm passage through the excurrent ducts (de Reviers, 1973). The quality and fertilising ability of the spermatozoa also increases when the frequency of semen collection is increased (de Reviers, 1973). If ejaculation does not occur over a two to three day period, then any spermatozoa stored in the vas deferens are reabsorbed (Leeson & Summers, 2000). Lake and Stewart (1978) suggested that semen should be collected three times weekly on alternate days in order to obtain maximum values of good quality semen. It has been shown that the number of semen doses over a six day period

increases linearly as the frequency of collection is increased from once every two days to twice daily, but in practice not all cockerels may be able to maintain full performance with such a demanding regime (Riaz *et al.*, 2004).

2.4.2 Insemination Technique

The timing of insemination relative to oviposition is an important factor determining the success of the insemination (Moore & Byerly, 1942, Brillard *et al.*, 1987). It is best if performed in the late afternoon, as it will minimize the possibility of oviposition interfering with sperm transport to the SST of the hens (Donoghue *et al.*, 1995). Chicken breeder hens at peak lay most of their eggs between the 5th and the 8th hour following the onset of the photoperiod (Brillard, 2003), and therefore AI should be performed after this.

In commercial operations, a common practice is to inseminate 100 million sperm per hen at weekly intervals, even though high levels of fertility have been observed following inseminations of only 50 million sperm cells per insemination dose (Etches, 1996). It is often convenient to inseminate 0.05 ml of diluted semen, because smaller volumes can be difficult to dispense and larger volumes tend to flow out of the vagina following insemination (Etches, 1996). Prior to insemination, the concentration of the semen therefore needs to be determined and the semen diluted in order to have the correct number of sperm in each insemination dose.

Semen diluents are buffered salt solutions designed specifically to maintain sperm fecundity as well as to dilute the semen so that a greater number of hens can be inseminated on a per male basis (Donoghue *et al.*, 1995). Semen dilution is important since poultry semen is viscous and highly concentrated (Donoghue & Wishart, 2000) and has a low volume (Bootwalla & Miles, 1992). Avian sperm are metabolically very active and diluents are designed to buffer the acidic products of their metabolism, especially during short-term storage (Etches, 1996).

The technique used for inseminating the hen involves applying pressure to the abdomen and everting the cloaca of the hen to expose the entry of the vagina (Leeson & Summers, 2000). The thighs of the hen are held between the thumb and the forefinger as the body rests in the open palm of the left hand. Gentle pressure is then exerted in a posterior

direction with the left hand as the tail is pressed in an anterior direction with the right hand, everting the cloaca of the hen (Etches, 1996). Semen is deposited two to four centimetres into the vaginal orifice concurrently with the release of the pressure on the hen's abdomen (Donoghue & Wishart, 2000). It is important that experienced operators perform the semen collection and artificial insemination, as inexperienced operators may use incorrect techniques that could result in lower fertility (Burrows & Quinn, 1938).

2.4.3 Commercial Use of Artificial Insemination

Artificial insemination is not as attractive in avian species as in some mammalian species as the use of fresh semen is required. Poultry spermatozoa are more susceptible to freezing damage due to their physiological characteristics (Donoghue & Wishart, 2000). The spermatozoa which retain their fertilising ability after freezing and thawing also appear to be unable to survive as long as fresh semen in the oviduct of the hen (Wishart, 1985). Turkey breeders have, however, been forced to use AI exclusively as a means of producing progeny because of the intense selection for breast meat output which has resulted in such major changes in body conformation that natural mating has become extremely difficult for the male (Donoghue & Wishart, 2000, Appleby *et al.*, 2004). In the duck and guinea-fowl, AI has also become successful as it helps in preventing unexpected drops of fertility during the late stages of the reproductive season (Brillard, 2006a). However, with the exception of a few countries (Indonesia, Philippines, Korea, India, China, and Taiwan) in which AI has become increasingly popular, natural mating remains the preferred method of breeding within the broiler breeder industry (Donoghue *et al.*, 1995, Brillard, 2006a). But, as broiler breeder stocks continue to become larger, and fertility declines (see Section 2.5), the potential for widespread application of AI increases and the benefits become economically attractive (Bilgili, 1989, Etches, 1996). The transition from natural mating of floor-pen breeders to AI of caged breeders will, however, involve a significant time and financial investment. Thus, the costs of successfully implementing an AI program, as well as the possible benefits of such a program need to be considered. The extensive use of AI in meat-type chickens may also be dependent on local or regional regulations for bird management (Brillard, 2006a).

Most of the costs involved with an AI program relate to the initial capital cost of cages (Leeson & Summers, 2000) and the increased cost of labour to collect and process semen

and inseminate breeder flocks (Etches, 1996). Another factor to consider is a greater incidence of footpad lesions that could occur if the cages are poorly designed (Leeson & Summers, 2000). It is, however, important to realise that the cost of labour could differ in different countries, possibly making AI more economically viable in those countries where the cost of labour is minimal. Also, the cages could be designed in such a way so as to minimise the occurrence of footpad lesions.

The economic benefits realised through implementation of an AI program include: increased feed savings (from 14 to 17 percent per hatched egg); greater selection pressure on males for economically desirable traits; better individual control of body weight gain if necessary; the need for fewer breeder males (male: female ratio with a well managed AI program is about 1:25 in contrast to 1:10 with natural mating); more settable eggs; higher and longer duration of fertility; and, the opportunity to separately optimize nutritional and environmental management programs for each sex (Bakst & Brillard, 1995). Caged housing would prevent fighting amongst the most aggressive birds and therefore reduce mortality (Etches, 1996). It would also make it possible to locate, and thus cull, hens and roosters that are performing poorly (Leeson & Summers, 2000). Artificial insemination could also be used as a means to prevent the age-related decline in fertility (see Section 2.5) by inseminating with the correct dose of viable spermatozoa throughout the reproductive period (Bramwell *et al.*, 1996, Kirby *et al.*, 1998).

In addition, the dwarf (*dw*) gene could be incorporated into the broiler breeder hen, and this would decrease feed costs as dwarf hens consume up to 30 percent less feed than their non-dwarf counterparts (Etches, 1996). Broiler breeder hen crosses carrying the *dw* gene also have the potential to tolerate *ad libitum* feeding while maintaining a high egg output (Heck *et al.*, 2004, Bruggeman *et al.*, 2005, Decuypere *et al.*, 2007). It has been suggested that these hens require only partial restriction (week 6 to 15 only) to maximise egg output (Decuypere *et al.*, 2007). Substituting standard with dwarf broiler breeders may therefore improve the welfare of commercial flocks of broiler breeders, resulting in favourable changes in behaviour, for example, reduced 'drinker-directed' behaviour (Jones *et al.*, 2004). It is, however, important to take into consideration that the male broiler offspring of the dwarf female parents are about 97 percent the size of the regular broiler, since the *Dw* gene is not 100 percent dominant to the *dw* gene (Leeson & Summers, 2000).

2.5 REPRODUCTIVE DETERIORATION

2.5.1 Effect of Age

Fertility in commercial flocks of broiler breeders typically increase to a peak at 30 to 40 weeks of age and declines thereafter (Hocking & Bernard, 2000). Some factors that have been suggested to contribute to this reduction include the physical condition of the male (Duncan *et al.*, 1990, Hocking & Bernard, 1997a, McGary *et al.*, 2002), sperm quality (Sexton & Renden, 1988, Hocking, 1989, Kirby *et al.*, 1998, Bowling *et al.*, 2003), and selection for body weight (Siegel & Dunnington, 1985). The frequency of mating behaviour in broiler breeders has also been found to decline with age (Duncan *et al.*, 1990, Hocking & Bernard, 1997a, McGary *et al.*, 2003). This is thought to be mainly as a result of altered musculo-skeletal conformation (e.g. increased breast muscle weight) due to selection for high yield, which may cause difficulty in achieving successful cloacal contact during mating (Hocking & Bernard, 1997a, McGary *et al.*, 2002, McGary *et al.*, 2003). Musculo-skeletal lesions, which were found to be associated with high body weights in broiler breeder males, have also been shown to have an adverse effect on fertility (Hocking & Duff, 1989).

An increase in the proportion of dead sperm in the ejaculates of older breeder males has been observed (Kirby *et al.*, 1998, Gumułka & Kapkowska, 2005) and semen volume and concentration of spermatozoa were found to be lower in older males (Sexton & Renden, 1988, Hocking, 1989, Rosenstrauch *et al.*, 1994, Gumułka & Kapkowska, 2005). A visual examination of the ductus deferens and testes of broiler breeders between the ages of 30 to 50 weeks indicated that all males appeared mature and normal (McGary *et al.*, 2002). However, Rosenstrauch *et al.* (1994) found that the seminiferous tubules in the testes of low-fertility aging roosters contained more Sertoli cell-spermatozoa complexes than did those of younger, more fertile males. It was suggested that the decline of fertility in aging roosters is related to changes in Sertoli cells that impair their ability to regulate the release of mature spermatozoa.

As mentioned before, testosterone is associated with sexual activity (Culbert *et al.*, 1977). The observed decline in mating frequency of broiler breeder males may therefore be related to a decline in circulating levels of testosterone as has been observed in aged broiler

breeder males (Sexton *et al.*, 1989a, b, Renden *et al.*, 1991). However, Hocking & Bernard (2000) observed relatively constant plasma testosterone concentrations in broiler breeder males from 36 to 69 weeks of age. It was also found that fertility, male display behaviour, and responses of the females to the males were not affected by the different ages of the males and females (Hocking & Bernard, 2000). It was suggested that there might be little difference in the fertility of male and female broiler breeders at different ages when body weight is adequately controlled to at least 60 weeks of age (Hocking & Bernard, 2000). A decline in fertility in commercial flocks is therefore likely to be the result of management failures, particularly in the control of male body weight (Hocking & Duff, 1989, Hocking & Bernard, 2000). This suggests that the decline in fertility in aging breeders could be linked to a decrease in the number of spermatozoa available for insemination. It may also be related to inefficient sperm transfer due to a behavioural or physical incapacity to complete copulations.

2.5.2 Female Contribution

In broiler breeder hens, the sequence length decreases rapidly from the time of peak egg production at 32 weeks of age to 62 weeks of age; increasing the incidence of 'first-of-sequence' eggs during the same period (Robinson *et al.*, 1990). It has been suggested that if the first egg of a laying sequence exhibited reduced fertility and decreased embryo viability, then the increased incidence of such eggs during the laying period might account for reduced fertility and hatchability seen with aging (Robinson *et al.*, 1991). Although Robinson *et al.* (1991) found that fertility and embryonic viability were not significantly influenced by the sequence position, the product of these two measurements (i.e. embryo production) was significantly ($P<0.05$) lower in the 'first-of-sequence' eggs than in subsequent eggs. In another study, flock age or time of oviposition (sequence position) were not found to have a significant effect on hatchability of fertile eggs or embryonic mortality (Zakaria *et al.*, 2005). Fertility did decline with flock age, but there were no differences due to the time of oviposition.

Older hens (55 to 63 weeks of age) have been found to have a significantly ($P<0.001$) shorter duration of fertility than younger females (32 to 41 weeks of age) (Brillard *et al.*, 1989). They are unable to retain spermatozoa in the SST for as long as young hens (Brillard, 1992). This was found to be a consequence of a faster release of spermatozoa

from the SST rather than a decreasing efficiency of sperm storage in the oviduct (Brillard, 2006b). It has been shown that older hens (39 to 40 weeks of age) require more spermatozoa than younger hens (31 to 32 weeks of age) to achieve high levels of fertility (de Reviers & Brillard, 1986). It has been suggested that old hens would benefit from duplicate inseminations, practiced on two consecutive days, followed by single inseminations of moderate doses (125 million sperm) practiced at weekly intervals (Brillard & McDaniel, 1986).

A comparison of results obtained after insemination of hens at 36 and 62 weeks of age (both groups of roosters were 36 weeks old then and were characterised by similar semen quality) showed lower values of sperm penetration of the PVL overlying the germinal disc at 62 weeks of age (Gumułka & Kapkowska, 2005). The percentage fertility as well as the duration of fertility of eggs obtained after insemination of hens at 56 weeks age was found to be similar, regardless of the age of the roosters (56 or 31 weeks) from which the semen was obtained (Gumułka & Kapkowska, 2005). Male age also did not appear to have a significant effect on the mean number of holes in the PVL overlying the germinal disc or in the duration of sperm penetration when hens aged 62 weeks were inseminated with ejaculates from either young or old males (Gumułka & Kapkowska, 2005). These observations indicate that female age had a greater effect than male age on decreasing the number of sperm penetration holes and the duration of fertility (Gumułka & Kapkowska, 2005). It is, however, important to note that the number of spermatozoa per insemination dose used in this study (125 x 10^6 spermatozoa on two consecutive days) was higher than recommended in practice, as the females were the focus of interest.

Interestingly, some other researchers have also found that the age of the hens appear to contribute more to the decrease in sperm penetration and fertility than the age of broiler breeder males, when the hens were inseminated with equal numbers of viable sperm throughout the reproductive period (Bramwell *et al.*, 1996, Kirby *et al.*, 1998). It would appear that the physiology of sperm from older males is relatively unaffected in their fertilising and sperm penetrating abilities, and that the observed age-related decline in fertility of naturally mated flocks is due more to physical problems as opposed to physiological restrictions of the male gamete (Bramwell *et al.*, 1996).

2.5.3 Spiking

A management technique that helps to sustain fertility performance in aging breeder flocks involves replacing older males with young males (also called "spiking") during the second half of the breeding season, and it has become current practice in many breeder operations worldwide (Brillard, 2004). It has been found that spiking is an effective procedure in activating old male reproductive activity and producing an increase in fertility (Casanovas & Wilson, 1999, Casanovas, 2002). This practice is, however, subject to criticism as there are a number of problems related to spiking (Casanovas, 2002). Some of these problems are listed below:

- Biosecurity could be breached if the replacement males were reared on other farms.
- Prior to spiking, the replacement males might not have contact with females, and their sexual behaviour and mating skills are likely compromised or at least require a learning period before they are able to mate efficiently.
- If the replacement males are too young or have not reached enough body size to successfully compete with the older males, their mating efficiency could be reduced and their contribution to the flock fertility delayed.
- If the replacement males are kept together after 25 weeks of age, aggression and high mortality will result, reducing the number of males available for spiking purposes.

As a solution to the spiking problems (mainly the biosecurity risks) and the observation that spiking with young males produced a stimulatory effect on the mating activity of old males (Casanovas & Wilson, 1999), the idea of "intra-spiking" was developed (Casanovas, 2002). This involves exchanging males of the same age between houses of the same farm or pens of the same house at 40 to 45 weeks of age. It disrupts the established hierarchy and stimulates mating activity of the original males similar to what is achieved by the young males in regular spiking. There is almost no cost involved as no extra males need to be housed and there are no biosecurity risks. Another advantage of this technique is that the added males will have mating experience and a similar conformation as the established males, which should allow them to successfully compete and mate from the beginning.

After conducting some trials with broiler breeders, Casanovas (2002) confirmed all of the above-mentioned advantages of intra-spiking. An increase in aggression and sexual interference was observed in the first two weeks post intra-spiking but, as opposed to regular spiking, no significant increase in mortality of either original or spiked males was observed. It was concluded that a double intra-spiking program at 40 and 48 weeks of age might be a good option to obtain a high and persistent mating activity stimulus and maintain significantly higher levels of hatchability for about 15 weeks in broiler breeder flocks. In order to minimise disruption of the social organisation within the flock, it is recommended that older males should be removed first (no more than 30 percent should be removed within a 24 h time period) and then the replacement males can be introduced after the lights are turned off (Donoghue *et al.*, 1995). It is, however, important to note that even though spiking has become current practice in many broiler breeder operations worldwide, it is now criticised as, in addition to the associated biosecurity risks and increase in the cost of hatched chicks, it may also dramatically disturb social and reproductive behaviour in breeder flocks (Brillard, 2006b).

2.6 MANAGEMENT OF THE BREEDER MALE

2.6.1 Body Weight Control

As already mentioned, reproductive traits and body weight (or growth rates) are negatively correlated in broilers (Siegel & Dunnington, 1985). Also, birds of heavy body weight lines have been found to be unable to assimilate feed in excess of their *ad libitum* intake, while lighter breeds were easily overfed and responded with an increased muscle and fat mass (Nir *et al.*, 1978). It was suggested that, unlike lighter breeds of chickens, which eat to meet their metabolic needs, heavy-breed chickens eat to near gut capacity. In a more recent study of male broiler and layer chickens, it was found that the hunger and satiety mechanisms in broilers have been changed compared with layer chickens (Bokkers & Koene, 2003). In contrast to layer chickens, where eating behaviour is controlled equally by satiety and hunger mechanisms, the eating behaviour of broilers was shown to be controlled more by satiety mechanisms than by hunger mechanisms. This suggests that broilers eat to their maximal physical capacity or some function of constrained feed intake, such as excessive heat production.

The broiler industry has, therefore, been forced to employ a number of management and nutritional schemes in order to control obesity and thus maintain the health and reproductive competence of broiler parent stock (Mench, 2002). This is typically accomplished by restricting the feed intake of the birds from a young age. The following section will review some of the research with respect to managing broiler breeder male fertility by nutrition and feeding practice.

2.6.2 Nutrition and Feeding

Various forms of feeding are used to limit growth and to improve fertility of broiler parents. These include limiting the quantity of feed offered daily, altering the nutrient density of the feeds, providing dietary supplementation, and using sex-separate feeding systems.

2.6.2.1 Restricted feeding – daily crude protein and energy allocation

Feed restriction is initiated when birds are one to three weeks of age (Mench, 2002). This ensures that the males are of a suitable size and capable of transferring the rapid growth characteristics, for which they have been selected, to their offspring. The optimum degree of feed restriction is, however, difficult to define due to strain differences and continual changes in the genetic composition of stocks by primary breeders (Robinson *et al.*, 1993). As broiler growth potential has increased in time, the degree of feed restriction needed to hold breeders on their target body weight profile has increased (Renema *et al.*, 2007). The following is an account of research in this area:

In a study of broiler breeder males on floors, it was found that restricted feeding had no significant effects on fertility or hatchability of the eggs produced by females that were artificially inseminated (Brown & McCartney, 1983). As expected, body weight gains were significantly ($P<0.05$) lower as the degree of restriction was increased. It was found that while birds fed the control level (corresponding to 26.18 g of CP and 1.92 MJ ME/b/d) had the largest testes as a percentage of body weight; the birds produced significantly ($P<0.05$) less semen on average over 24 weeks than males being fed 15 percent less feed. It was concluded that males on moderate feed restriction produced the highest volume of semen. In a later experiment, broiler breeder males in cages also exhibited better reproductive

performance when subjected to a more severe feed restriction than recommended by the breeder (Brown & McCartney, 1986). It was suggested that males housed in individual cages require 19.72 g of CP and 1.44 MJ ME/b/d for body weight maintenance.

However, significant ($P<0.05$) reductions have been observed in semen volume, sperm cell numbers per ejaculate and testicular weights with more severe feed restriction (corresponding to 11.92 g of CP and 1.21 MJ ME/b/d) of broiler breeder males in cages (Buckner *et al.*, 1986). It has been suggested that reproductive functions of broiler breeder males in cages are more sensitive to underfeeding than overfeeding (Sexton *et al.*, 1989a). A detrimental effect on spermatozoa production was observed when the feed intake of broiler breeder males in cages was restricted (to 75 percent of the breeder-recommended levels), whereas effects of *ad libitum* feeding were beneficial. A higher percentage of the *ad libitum*-fed males were producing semen, with greater volumes and concentrations, throughout the experiment. This indicates that the level of feed intake recommended by the breeder was possibly too low. Also, that levels of restriction recommended by poultry breeders serves more to allow for better mating behaviour than altering semen quality. In another study of broiler breeder males, it was found that giving the males 13.2 g CP/b/d and 1.27 MJ ME/b/d during the reproductive period depressed the reproductive performance (Cerolini *et al.*, 1995). An increase in the daily quantity of food improved the fertility. The optimal daily food supply for the best reproductive performance was found to corresponding to 15.6 g CP/b/d and 1.50 MJ ME/b/d.

It appears that the difference in response to feed restriction, as observed by Brown and McCartney (1983, 1986) and the above authors, might be as a result of the level of feed restriction to which the birds were subjected. Buckner *et al.* (1986), Sexton *et al.* (1989a) and Cerolini *et al.* (1995) used more severe levels of feed restriction than what was used in the studies of Brown and McCartney (1983, 1986). In another study, a quadratic treatment effect on motility, vigour and fertility of spermatozoa was observed when energy intakes ranged between 1.21 and 1.55 MJ ME/b/d (Borges *et al.*, 2006a). Based on these observations, an intake of 1.45 MJ ME/male was recommended to meet the requirements of male broiler breeders from 26 to 61 weeks of age (Borges *et al.*, 2006a).

No differences in early fertility have been observed between males weighing 3.0 or 3.5 kg at the start of the mating period, or between males gaining 1.0 or 1.5 kg body weight from

mating to 60 weeks of age (Hocking & Bernard, 1997a). It was suggested that broiler breeder males could be less severely restricted than is currently practiced in commercial flocks with possible benefits to their welfare. Brake (2003) suggested that an appropriately controlled feed allocation rather than severe restriction is required. It is important to note that excessive feed restriction of males during part or all of the growing period has been associated with decreased early fertility (Lilburn *et al.*, 1990). It has been reported that severe feed restriction between 42 to 72 percent of free choice had a detrimental effect on the fertility of Rhode Island Red males (Parker & McSpadden, 1943). It is likely that overly severe restriction has actually caused fertility problems due to reduced mating activity as a result of caloric deficiencies (Brake, 2003).

This may help explain the observations of Hocking (1990) who performed experiments with broiler breeder males in floor pens with natural mating during the breeding period. A curvilinear relationship was found between fertility and body weight. The shape of the curve is such that if body weight is too low or too high, optimum fertility is not attained. It was observed that underweight males were not physiologically sufficient (low testes weights with few or no spermatozoa on histological examination of the testes) while overweight males were often incapable of completing the mating process. It was suggested that there is an optimum body weight for maximum fertility, which changes with age, and that restricted control of body weight should allow an increase in body weight throughout the breeding period.

Brake (2003) conducted a study to examine this relationship. It was concluded that male body weight was better controlled and fertility improved when the male feed allocation was increased slowly rather than decreased. It was suggested that the daily feed allocation be increased at least 1 g every three to four weeks during the breeding period such that the male body weight increases slowly but consistently and remains within limits established by practical experience and known to be associated with good fertility. Behavioural problems, particularly egg eating, by males at lower body weights (Hocking, 1990) support the suggestion that males should be permitted to grow by allocating increasing quantities of food throughout the breeding period.

McGovern (2002) also suggested that males benefit from a consistent and gradual increase in body weight. The effect of body weight loss from 35 weeks of age on reproductive

characteristics of broiler breeder males was studied. The body weight loss resulted in a 14.7 percent lower fertility from eggs 8 to 14 d post-insemination compared to the standard gain treatment at 58 weeks of age even though semen volume and concentration were not negatively affected. In a study of two lines of broiler breeder males, no evidence was found to show that males, that were fed on an increasing quantity of feed, produced more semen or a greater concentration of spermatozoa than males on a fixed daily feed allocation (Hocking & Bernard, 1997b). The increase in fertility observed in broiler breeders gaining body weight gradually during the breeding period (Hocking, 1990, McGovern, 2002, Brake, 2003) in the absence of an effect on semen quantity and quality (Hocking & Bernard, 1997b, McGovern, 2002) might suggest that the additional feed supplied the males with the nutrients required to mate more effectively than males subjected to constant or decreasing feed allocation. It has been suggested that a rising plane of energy is necessary to maintain body weight and testicular function in broiler breeder males (Hocking, 1989), therefore the additional feed allocation might also have had an affect on the testicular function and thus the fertilising potential of the sperm, even if no differences in volume and concentration were observed.

Further work was done to investigate the effect of feeding program on the body weight and fertility of broiler breeder males (Romero-Sanchez *et al.*, 2007a, b, Romero-Sanchez *et al.*, 2008). It was observed that males that received rapid feed increases during the late rearing and early production period (16 to 26 weeks of age) were unable to maintain their body weight from 32 weeks of age and had a greater decrease in fertility after 42 weeks of age than males that received gradual feed increases during the same period (Romero-Sanchez *et al.*, 2007a, b). It was suggested that the heavier males required a higher ME intake later in production to support a modest body weight gain and maintain fertility (Romero-Sanchez *et al.*, 2007a, b). This idea was supported by the observation that a 5 g increase in the daily feed allocation (an additional 0.06 MJ ME/b/d) at 49 weeks of age was sufficient to restore fertility (Romero-Sanchez *et al.*, 2007a). It is, however, important to note that prior to the feed increase, 1.38 MJ ME/b/d of energy was supplied from 26 weeks of age onwards, which is less than what was recommended by Cerolini *et al*. (1995) and Borges *et al*. (2006b). Romero-Sanchez *et al*. (2007b, 2008) found that breeder males given an increasing feed allowance throughout the production period produced a significantly ($P<0.05$) smaller decrease in fertility than males maintained on a constant feeding program; and it was suggested that feeding programs should be designed to maintain body

weight gain rather than focussing on the attainment of some fixed body weight. These findings are in agreement with that of Hocking (1990), McGovern (2002) and Brake (2003). Interestingly, the benefits of providing sufficient nutrients to maintain mating behaviour and libido of breeder males was shown to not only increase the fertility of breeder flocks, but also to have positive effects on the overall genetic potential of the broiler progeny for growth and feed efficiency (Romero-Sanchez *et al.*, 2008).

The two most commonly used commercial restriction programmes are skip-a-day (SAD), in which birds are fed twice the breeder-recommended amount of feed on alternate days (Vaughters *et al.*, 1987, Mench, 2002); and limited every day (ED), in which the breeder-recommended amount of feed is fed daily. Some research has been conducted in order to evaluate the effect of these feeding schedules on broiler breeder males.

It has been suggested that ED feeding is preferable to SAD feeding for body weight gains and feed efficiency (Vaughters *et al.*, 1987). This is because breeder males fed ED were found to have gained more than those fed on alternate days on nearly identical amounts of feed (as SAD treatment birds were fed *ad libitum* in this trial and feed intake was recorded). Feed savings resulted from ED feeding, while no increase in bird variation was observed. Fertility was also found to be better in birds fed ED, but semen volumes and concentrations were not affected by the feeding schedule. Additional benefits of ED feeding that were observed by Vaughters *et al.* (1987) are a reduction in litter consumption (decreasing exposure to disease and parasites), and less aggression. These findings suggest that there are also welfare benefits in feeding the males ED rather than on alternate days.

The findings of the study mentioned above were confirmed by those reported in another study (Sexton & Renden, 1998). In this study, birds that were fed daily were also found to have a higher feed efficiency than SAD-fed birds. It is also interesting to note that the age at sexual maturity was significantly delayed (by 10 days) in SAD-fed birds compared with those fed daily. However, testes weights were significantly reduced at 34 weeks of age in birds fed daily, when compared with SAD birds, possibly because sexual maturity was hastened in these birds. As mentioned above, Vaughters *et al.* (1987) did not observe any effect of feeding schedule on semen characteristics from 24 to 27 weeks of age, but Sexton & Renden (1998) found that males of the SAD group produced significantly more semen with higher spermatozoa concentrations than the group of birds that were fed daily. The

differences in semen production did, however, not appear until the birds were 28 weeks and older. This difference in semen production could possibly be explained by the difference in testes weights, as observed between the two groups of birds at 34 weeks of age.

It is evident, from these reports, that feeding schedule has an effect on body weight gains and feed efficiency of broiler breeders. The prolonged effects on semen characteristics and fertility are, however, not very clear since these studies were terminated before the end of the breeding period. It is also important to consider the welfare of these birds, when deciding on a feeding schedule, as the birds will already be restricted with regards to the amount of feed allocated.

2.6.2.2 Nutrient density

This following section will briefly discuss the use of low density diets in an attempt to improve the welfare of broiler breeders that are restricted. Despite the positive influences on health and reproduction, there is evidence that feed restriction has negative effects on welfare. Broiler breeders show evidence of physiological stress as well as increased incidence of abnormal behaviours, and are also chronically hungry (Mench, 2002). Restricted birds have been found to show hyperactivity, stereotyped pacing before feeding time, and stereotyped drinking and pecking at non-food objects after feeding (de Jong *et al.*, 2002). It has been suggested that low-density diets could be used as an alternative feeding method as it may improve broiler breeder welfare during rearing (de Jong *et al.*, 2005, Sandilands *et al.*, 2005), especially if eating behaviour relies more on satiety mechanisms than on hunger mechanisms (Bokkers & Koene, 2003).

In an experiment in which broiler breeder females were fed diets diluted with ground oat hulls from 0 to 56 weeks of age, diet dilution was found to improve the well-being of feed-restricted broiler breeders (Zuidhof *et al.*, 1995). Improvements in behaviour have also been observed in broiler breeders fed a diluted diet (10 to 13 percent wheat bran plus additional dilution with 10 percent sand, starting at the 10th week of life) *ad libitum* (Heyn *et al.*, 2008). However, these birds weighed far more than the breeders' recommendations, and it was suggested that appetite suppressants such as calcium propionate or phenyl propanolamine needs to be added to the diluted diet before it would be an effective

alternative to quantitative feed restriction. Sandilands *et al.* (2005) found that broiler breeder females can be successfully limited in growth rates by qualitative food restriction during rearing and observed that this resulted in significant changes of behaviour that suggests improvements to bird welfare. During rearing (1 to 20 weeks of age), birds were either fed limited amounts of standard basal diets (i.e. quantitative restriction), or *ad libitum* diets consisting of standard basal diets with gradually increasing levels of calcium propionate and a constant level of oat hulls (i.e. qualitative restriction). Thereafter, all the birds were fed on a conventional quantitative restriction regime. The differences in behaviours that were observed between treatment groups during rearing disappeared during lay when all the birds were fed a similar amount of food.

From the above-mentioned studies, it is evident that the welfare of restricted-fed broiler breeders could be improved by the use of qualitative feed restriction, during the rearing period, or for the entire cycle, provided that the feed is properly formulated to meet the requirements of these birds at all times. However, finding a diluent that can be used in large enough quantities, or that does not make some nutritional contribution to the diet, is difficult and may also be costly (Mench, 2002). It is also important to ensure that the diluent selected will not have adverse affects on the health or overall production of the birds.

2.6.2.3 Selenium and vitamin E supplementation

Spermatozoa of avian species contain high proportions of long-chain polyunsaturated fatty acids (PUFA) (Surai *et al.*, 1998) to maintain membrane fluidity and flexibility; properties needed for sperm motility as well as sperm fusion during fertilisation (Surai, 2002). However, these high concentrations of PUFA in avian spermatozoa make them vulnerable to lipid peroxidation, which is considered to be an important factor in male infertility (Surai, 2002).

Selenium (Se) is an essential component of glutathione peroxidase (GSH-Px); an enzyme that aids in protecting cellular contents and sub-cellular membranes from oxidative damage by destroying peroxides before they attack these membranes (Surai, 2000, Rutz *et al.*, 2005). It has been suggested that dietary Se supplementation may have an important

impact on poultry reproduction at the level of sperm formation, sperm storage, and in the hatching egg through increased protection from oxidative damage (Renema, 2004).

Vitamin E is a specific lipid soluble antioxidant in the membrane (Rutz *et al.*, 2005). It has been suggested that an increased dietary intake of vitamin E produces beneficial changes in the antioxidant capacity and lipid profile of poultry semen, maintaining the structural integrity and fertilising capacity of spermatozoa (Surai *et al.*, 1997). A relationship exists between vitamin E and Se and it has been suggested that an increased dietary supplementation of both of these nutrients could be beneficial to breeder males in maintaining the integrity of the sperm membranes (Surai *et al.*, 2000).

2.6.2.4 Sex-separate feeding

Equipment that allows for sex-separate feeding of broiler breeders has been developed and is currently in use in the industry. These systems are designed to ensure that the grill covering the female feeder is too fine to allow the males access to the feed, and to ensure that the male feeder is high enough to prevent the hens from reaching the male feed (Etches, 1996). Prior to the adoption of this feeding method, adult male broiler breeders were not actively managed to achieve optimum reproductive potential (Robinson *et al.*, 1993). This system permits males and females to be fed different amounts of feed, and even different feed rations, while housed together. Sex-separate feeding of broiler breeders has been found to allow for better control of the male body weight and significant ($P<0.05$) increases in fertility and hatchability have also been observed (Andrews *et al.*, 1988, Fontana *et al.*, 1990).

2.7 CONCLUSIONS

Broilers have been selected to grow at a very rapid pace, and will eat to near gut capacity, when fed *ad libitum*. Broiler breeders have the same genes for fast growth as their offspring, however, if allowed to eat *ad libitum*, a number of complications can occur, which would result not only in a decrease in fertility, but also impair health and mating ability.

There is an age-related decrease in fertility of breeders. Various management strategies have been suggested to maintain fertility. However, a number of them are labour intensive and would require a significant financial input. One management practice, that is used extensively, is to limit the growth of broiler breeders by feed restrictions. The behaviour of restricted-fed birds have indicated that these birds were under a lot of stress. From a welfare point of view, *ad libitum* feeding would not be of any advantage to the birds, as there are a number of health risks in birds that are overweight. The effect of various levels of feed allocation on broiler breeder males have been reported. However, as the results in the literature are contradictory, further research is needed to establish firm conclusions on the effect of different dietary nutrients on broiler breeder male reproductive characteristics. Therefore, the aim of the current experiment was to determine the possible benefits of feeding breeder males diets with lower crude protein levels than what is recommended for breeder females, in an attempt to better manage body weight and to sustain or improve fertility. The possible benefits of dietary supplementation of broiler breeder male diets with additional vitamin E or Selenium was also investigated.

CHAPTER 3

SEMEN COLLECTION AND ARTIFICIAL INSEMINATION TECHNIQUES

3.1 INTRODUCTION

Artificial insemination and semen quality analysis in poultry had not been performed much at the University of KwaZulu-Natal in recent years before this experiment. It was therefore considered necessary to practice and perfect various experimental techniques before any trial work was performed, and thus, a pilot study was undertaken to ensure fertile eggs after AI, as well as to obtain practice in feed formulation and mixing of feed.

Results from trials investigating the effects of crude protein (CP) on male breeder fertility have not been consistent. A number of researchers found no significant effect of dietary CP content on fertility of broiler breeders (measured by candling eggs after 18 days of incubation) (Fontana *et al.*, 1990, Hocking & Bernard, 1997a), whereas Hocking (1990) observed a negative effect of high CP on fertility. In some cases, no significant effects of CP level in the feed on semen quality traits were observed (Wilson *et al.*, 1987a, Wilson *et al.*, 1987b, Hocking, 1989, Revington *et al.*, 1991, Zhang *et al.*, 1999). However, other researchers found that a high protein diet adversely affected semen quality (Hocking & Bernard, 1997b, Selvan, 2007) and negatively affected the proportion of broiler breeder males producing semen (Wilson *et al.*, 1987a, Wilson *et al.*, 1987b, Hocking, 1989). Borges *et al*. (2006a) observed a decrease in the reproductive performance of breeder males at levels of both deficiency or of excess of protein intake.

It is important to determine whether there are any advantages, such as improved fertility, in the use of specific male breeder rations during the reproductive period. Therefore, the objectives of this preliminary trial was to perfect AI techniques and investigate the effect of different levels of dietary inclusion of CP on semen quality and fertility of broiler breeder males, while keeping energy levels of the feeds constant.

3.2 MATERIALS AND METHODS

Nine Cobb broiler breeder males were obtained from the Cobb breeder house at the poultry section on Ukulinga Research Farm where they had been housed together with broiler breeder females of the same strain. These males were removed from the breeder flock at 53 weeks of age in order to be used in this preliminary trial. After being removed from the breeder house, the males were housed in individual cages on a litter floor in an open-sided house, where they remained for the entire duration of the trial. Each cage had a nipple drinker and the birds were provided with *ad libitum* access to water.

Two basal diets (a summit and a dilution diet) were formulated. The summit diet was formulated according to the breeder recommendations for males over 155 days (Cobb-Vantress, 2005). The dilution diet was formulated according to the same nutrient levels, but the objective function was to "minimise total essential amino acids" and ingredients with almost negligible amounts of protein contents were used. In doing this, a dilution diet was created that contained very little crude protein but similar amounts of all other nutrients as the summit diet. The ingredient contents of the two basal diets are given in Table 3.1.

Table 3.1 *Dietary ingredient contents (g/kg) for the two basal diets used in the experiment*

Ingredient	Summit	Dilution
Yellow maize fine	671.2	
Starch		650.0
Sand		193.6
Wheat bran	156.6	
Soybean 50	136.4	
Sunflower husks		72.2
Sunflower oil		30.0
Limestone	18.0	16.3
Monocalcium phosphate	10.7	17.2
Potassium carbonate		11.2
Sodium bicarbonate	3.5	3.3
Salt	2.2	2.8
Choline chloride 60%		1.9
Vitamin & mineral premix	1.5	1.5

The nutrient compositions of the two basal diets were calculated in order to determine the ratio in which to blend them to get the required crude protein levels in the experimental diets. The two basal diets were mixed and then blended together in various proportions to form the three experimental diets (Table 3.2).

Table 3.2 *The percentage compositions of the three experimental diets*

Experimental diet	Protein level	% Summit	% Dilution
1	Low	65	35
2	Control	85	15
3	High	95	5

After blending the two basal diets, the nutrient compositions of the three experimental diets were calculated (Table 3.3). Since this was a preliminary trial, the feeds were not analysed for their respective nutrient contents.

According to the breeder management guide, adult males can be kept very active and in good condition with 1.548 to 1.590 MJ ME/b/d (Cobb-Vantress, 2005). For the purpose of calculating required feed intake, an average ME requirement of 1.569 MJ/b/d was used. Taking the ME content of the experimental diets into consideration, the feed intake required in order to meet the daily ME requirement was calculated to be 136.08 g/b/d. Each male was thus fed 136 g/d of its respective experimental diet for the duration of the trial. Therefore, the males on the low, control and high CP treatments were receiving 13.42, 17.31 and 19.27 g of CP/b/d respectively. Males were randomly allocated to each of the dietary treatments.

Since the males were kept with the broiler breeder females prior to being placed into individual cages, training to produce semen in response to the abdominal massage method (Burrows & Quinn, 1937) was required. The feathers around the cloaca of each male were clipped in order to minimise contamination of the semen during collection. The males did not yield much semen and it was therefore necessary to pool the semen from the males on the same treatment prior to insemination. After the males had been on experimental diets for a period of a week, semen was used for artificial insemination of commercial layer hens. Care was taken to avoid collecting the transparent watery fluid that is sometimes produced in the fowl at ejaculation in response to massage to prevent dilution of the

sample (Lake & Stewart, 1978). Semen was collected into small plastic flip top vials. After collection, a known volume of semen was removed from the pooled semen sample with a syringe and diluted in a 1:1 ratio with the semen diluent, Tyrodes solution (Appendix A).

The day on which insemination was performed was referred to as Day 0 (D0). Eggs were collected on D2, D8 and D14 from all the inseminated hens and stored in a cold room for no more than two weeks before being assessed.

The eggs were brought to room temperature, cracked open, and the yolks were separated from the albumen. Excess albumen was removed by rolling the yolk on a paper towel. A square of approximately 1 x 1 cm of the perivitelline layer situated around the germinal disk area was then cut and removed. This piece of membrane was rinsed in phosphate-buffered saline (PBS) to remove adherent yolk, before being stretched out on a glass microscope slide. It was then stained with a 1 μg/ml solution of diamidinophenylindole (DAPI) in PBS and covered with a cover slip (Wishart, 1987). The slides were examined under fluorescence microscopy on the same day they were prepared. The sperm nuclei embedded in the outer perivitelline layer ($OPVL_{sperm}$) in 20 randomly-chosen fields of view (each 177687.19 μm^2) were counted and totalled. These numbers were then expressed as sperm per mm^2 of membrane.

Artificial insemination was performed in the late afternoon, when most of the laying hens had already laid their egg for the day. The insemination was performed once a week, for two weeks on different hens. Initially, plastic syringes were used to inseminate each hen with a volume of 0.1 ml of diluted semen. It soon became apparent that it was difficult to work with such small volumes of semen using a syringe. Also, no sperm nuclei were visible in the OPVL of any of the eggs collected after the first AI. A factor that may have contributed to this is that the semen might not have been deposited far enough into the vagina since the tip of the syringe was very short.

A thin flexible tube was then attached to the tip of the syringe in order to lengthen the tip without possibly inflicting damage on the hen's vagina. This change did not seem to be effective though, as there were still no visible sperm nuclei in any of the eggs that were assessed. Some of the semen was observed to remain inside the tube, after insemination. This was possibly due to a lack of adequate pressure to force the semen out of the tube.

After experimenting with these techniques, it was found that an Eppendorf pipette could be used to effectively deposit an accurate volume of semen into the vaginal orifice of the hen. A new pipette tip could also be used each time in order to prevent any contamination of the semen samples.

Table 3.3 *The calculated nutrient contents (units specified) of the three experimental diets*

Nutrient	Unit	Diet 1	Diet 2	Diet 3
AMEn_adult	MJ/kg	11.53	11.53	11.53
EE	MJ/kg	10.34	10.32	10.31
Crude protein	%	9.87	12.73	14.17
Dry matter	%	90.50	88.29	87.18
Lysine	%	0.40	0.51	0.57
Methionine	%	0.15	0.19	0.22
Cysteine	%	0.16	0.21	0.24
Methionine + cystine	%	0.31	0.41	0.45
Threonine	%	0.31	0.40	0.45
Tryptophan	%	0.09	0.11	0.13
Arginine	%	0.56	0.72	0.80
Isoleucine	%	0.35	0.46	0.51
Leucine	%	0.86	1.12	1.25
Histidine	%	0.25	0.33	0.37
Phenylalanine	%	0.39	0.51	0.56
Tyrosine	%	0.32	0.42	0.46
Phenylalanine + tyrosine	%	0.71	0.92	1.03
Valine	%	0.43	0.56	0.62
Ash	%	3.80	4.06	4.18
Crude fibre	%	3.53	3.52	3.52
Crude fat	%	3.37	3.38	3.38
Calcium	%	0.90	0.90	0.90
Available phosphorous	%	0.43	0.43	0.43
Sodium	%	0.20	0.20	0.20
Chloride	%	0.20	0.20	0.20
Potassium	%	0.70	0.70	0.70
Linoleic acid	%	1.84	1.81	1.79
Choline	mg/kg	990.20	990.27	990.30

In order to examine some semen quality traits, semen was collected once a week for a period of three weeks, beginning one week after the last insemination was done. Unlike the semen that was used for AI purposes, the semen was not pooled and thus each male

represented a replicate of its respective treatment. It is important to note, however, that not every male yielded semen on all of the collection days.

- **Morphology**

Immediately after semen collection, a drop of undiluted semen was placed in 100 µl of 3 percent glutaraldehyde and mixed lightly. This is done in order to fix and preserve the spermatozoa in their state at ejaculation. These samples were then kept to be examined at a later date. A drop of the sample was placed onto a microscope slide and gently covered with a cover slip before being examined with a light microscope at 40 times magnification. Three hundred spermatozoa on each slide were counted at random and defects were noted and then calculated as a percentage of total sperm observed. The following defects could be noted: sperm bent at head-midpiece or midpiece-tail junctions, knots or swelling at head-midpiece junctions or in the head region, and coiled heads (Lake & Stewart, 1978, Alkan *et al.*, 2002).

- **Concentration**

Prior to semen collection, solutions containing Eosin and Nigrosin were prepared for each semen sample (Appendix B). A known volume of undiluted semen (either 50 or 100 µl, depending on the volume of semen yielded by the bird) was placed within the Eosin and Nigrosin solution and mixed well by rotating the bottles gently. A small amount of the mixture was then used to fill the two sides of a haemocytometer. Spermatozoa were counted in 10 random blocks on either side of the haemocytometer at 40 times magnification using a light microscope. This data, together with the corresponding mass motility scores, were then used in a formula (Appendix C) to calculate the number of live sperm per ml of semen.

- **Motility**

After removing the semen needed for the concentration and morphology assessments, the remainder of each semen sample was immediately diluted with Tyrodes solution and placed aside before proceeding to the next male. Once semen had been collected from all

of the males, the motility assessment was done. A drop of the diluted semen sample was placed on a pre-warmed slide and gently covered with a cover slip. A light microscope at 40 times magnification was used to examine the sample. Three random areas on the slide were examined in order to score the percentage progressive motile normal sperm, the percentage abnormal motile sperm and the percentage dead sperm. Care was taken to minimise the time between collecting and analysing the semen motility, as poultry sperm are very active (thus producing lots of waste products) and will start to die in less than an hour (Etches, 1996).

The broiler breeder males were weighed individually at the beginning of the trial and seven weeks thereafter. An electronic scale was used and the males were placed head-first inside a cone during weighing in order to restrain them and to avoid injuries.

Statistical Analysis

All data were analysed using *GenStat 11th Edition* (Lawes-Agricultural-Trust, 2008). The data were subjected to a general analysis of variance (ANOVA) with an unbalanced treatment structure. This type of ANOVA was used due to the different number of birds from each treatment that yielded semen on the collection days. All means were compared using the appropriate least significant difference (LSD) values at 5 percent level of significance.

3.3 RESULTS AND DISCUSSION

As mentioned above, no $OPVL_{sperm}$ nuclei were visible in any of the eggs collected after the first two inseminations. It was suggested that an Eppendorf pipette could be used to deposit the semen into the vaginal orifice of the hen. This could potentially be more accurate than a syringe, not only because of the longer tip, but also due to the design of the Eppendorf pipette that allows for a greater ability to accurately measure out and release most of the semen. Another factor that could have contributed to the lack of $OPVL_{sperm}$ visible under fluorescence microscopy is that the period of time that elapsed between the staining and examining of the slides could have been too long. Also, the DAPI stain that was used could have lost its fluorescence due to exposure to external light. It was decided

that, in future, the slides would be kept in a light tight container and that the slides would be examined soon after being prepared. However, before these alterations could be implemented, some of the breeder males were lost due to theft. This part of the trial therefore had to be ended earlier than planned as there were not enough replicates of males of each treatment.

No significant differences were found for the number of live sperm per ml of semen sample between the CP treatment groups (Table 3.4). However, the control CP group had a larger amount of live sperm per ml of semen than the other treatment groups. This corresponds to the higher percentage normal spermatozoa observed in this group (Table 3.5), but it is evident from Table 3.4 that a large proportion of motile sperm in this group were moving in an abnormal manner. A possible explanation for this may be that the morphological shape and physiological activity of some of the spermatozoa may have been altered between the time of semen collection and motility assessment because the sample of semen that was used to determine sperm morphology was fixed immediately after collection, whereas the motility was only assessed once semen was collected from all of the males. It is also possible that there were abnormalities at the time of production which could have been due to diet but also age or other environmental factors.

Sperm that were bent or that had a knot at the head-midpiece junction were the most prevalent morphological defects observed in this study. The midpiece of poultry semen is a very sensitive region as it deteriorates quicker than other regions (Maeda *et al.*, 1986) and it has been suggested that spermatozoa are bent due to a weakening of the midpiece (Lake & Stewart, 1978). These sperm are motile when viewed in drops of semen placed under the microscope, but are infertile (Lake & Stewart, 1978). This would explain why the high CP group did not have a significantly lower number of live sperm per ml of semen, despite their lower percent normal spermatozoa.

Table 3.4 *Mean values (± s.e.m.) of motility, semen concentration and numbers of live sperm per ml of semen obtained from Cobb broiler breeder males fed different amounts of crude protein per day*

Treatment (CP level)	Motility (%)			Concentration sperm/ ml ($x10^6$)	Number of live sperm/ ml ($x10^6$)
	Progressive normal motile	Abnormal motile	Non-motile (dead)		
Low	75.7 ± 8.1	6.8 ± 2.9	17.5 ± 6.4	20.9 ± 31.2	18.9 ± 29.3
Control	74.4 ± 7.6	14.4 ± 2.7	11.3 ± 6.0	78.9 ± 29.2	73.8 ± 27.4
High	81.7 ± 8.8	10.0 ± 3.2	8.3 ± 7.0	55.0 ± 33.7	50.1 ± 31.6

Table 3.5 *Mean values of morphology (± s.e.m.) of semen obtained from Cobb broiler breeder males fed different amounts of crude protein per day*

Treatment (CP level)	Morphology (%)					
	Normal	Bent	Coiled heads	Knots at head-midpiece junction	Swelling in head	Swelling at head-midpiece junction
Low	94.7 ± 0.8	2.7 ± 0.7	0.4 ± 0.1	0.7 ± 0.2	0.8^a ± 0.1	0.7 ± 0.2
Control	96.5 ± 0.8	1.9 ± 0.7	0.1 ± 0.1	0.9 ± 0.2	0.3^b ± 0.1	0.3 ± 0.2
High	94.2 ± 0.9	3.8 ± 0.7	0.2 ± 0.1	1.2 ± 0.2	0.4^b ± 0.1	0.3 ± 0.2

[a,b] Means in a column followed by different superscripts are significantly different at 5 percent level of significance

Spermatozoa that have coiled heads are in the late stage of disintegration (Lake & Stewart, 1978). There was no significant difference for the mean percentage of coiled heads observed between the CP treatment groups. This indicates that the amount of time that elapsed between the collections and fixing of the semen samples was similar for the various CP treatment groups. It may also indicate that the period for which the spermatozoa were stored in the vas deferens was similar for the males on all the treatments.

According to Lake & Stewart (1978) the occurrence of bulbs (swelling) in either the head or at the head-midpiece junction appears to represent sperm that are malformed at the spermatid stage of their production in the testes. The percentage spermatozoa observed to have swelling in the head region was significantly ($P<0.05$) higher in the low CP group. The mean percentage of sperm that were observed to have swelling at the head-midpiece junction was also higher in the low CP treatment group. This may indicate that one or more of the birds in this CP group may have been producing malformed spermatozoa, possibly due to an age-related decline in testicular functioning or due to malnutrition. The lower

concentration of sperm per ml and number of live sperm per ml observed in this treatment group may also support these ideas. However, there was no significant difference in body weight of the males in the various CP treatment groups.

It has been suggested that the vagina and the uterovaginal junction are selective areas of the oviduct preventing spermatozoa with abnormal morphology, poor motility or sluggish metabolism from reaching the site of fertilisation in the hen (Ogasawara *et al.*, 1966, Bakst *et al.*, 1994). It has also been recommended that more than one semen characteristic needs to be assessed, in order to predict the potential fertility of broiler breeder males (Wilson *et al.*, 1979). For these reasons, semen morphology was examined and the motility and concentration of spermatozoa in the semen samples were used in order to determine the number of live sperm per ml of semen.

Wishart (1987) developed a non-invasive technique to determine the density of spermatozoa embedded in the outer perivitelline layer of oviposited eggs. This procedure has been shown to be a reliable biological assay for the evaluation of semen quality and sperm-storage capacity of the oviduct at the time of insemination (Brillard & Bakst, 1990). It provides estimates of both the duration of fertility (Wishart, 1987) and the number of spermatozoa from a single insemination that were selected by the SST for storage (Brillard & Bakst, 1990). The quantification of $OPVL_{sperm}$ can also be used to predict the fertility of an egg (Brillard & Antoine, 1990, Wishart, 1997). Semen from the breeder males was thus used to inseminate laying hens in order to look at the ability of the spermatozoa to survive in the hen and to possibly predict the fertilising ability of the semen.

It is evident from the results of this study that, except for the percentage spermatozoa with abnormal swelling in the head region, there were no significant treatment effects with regards to the level of CP in the diets of the breeder males. It does, however, appear that the birds performed better on the control level of CP, which corresponded to an intake of 17.31 g of CP/b/d. Borges *et al.* (2006a) recommended that an intake of 16.9 g of CP/b/d should be adequate to meet the CP requirements of male broiler breeders from 27 to 61 weeks of age. They observed a decrease in reproductive performance (semen volume, sperm concentration, motility, spermatozoa vigour and fertility) at extreme levels (deficiency or excess) of protein intake. Therefore, the poorer semen quality observed in the males on the low or high CP treatments (receiving 13.42 and 19.27 g of CP/b/d

respectively) may be explained by a CP intake below, or in excess, of a certain requirement for maximum fertility. Therefore it would be useful to determine this ideal CP intake and compare it to recommended levels and levels required for maintenance.

Spermatogenesis in the mature chicken occurs continually and newly formed spermatozoa may appear in the ejaculates after only 13 to 15 days (Howarth, 1995). The males were given the treatment feeds for a period of three weeks before semen analysis commenced, thus it is possible that the dietary CP may have had an effect on semen quality.

3.4 CONCLUSIONS

This was an important experiment to conduct, as it highlighted a number of factors that needed to be considered in future trials. It also enabled the acquisition of invaluable skills that could potentially be applied to further work. Most of the results indicated that there were no significant treatment effects with regards to the level of CP in the diets of the breeder males. It does, however, appear that the birds performed better on the control level of CP, which corresponded to an intake of 17.31 g of CP/b/d. It is important to note that the males were on the CP diets for a very short period of time, towards the end of their production period. Thus, the relationship between dietary CP intake and breeder male semen quality requires further investigation, particularly during the early and peak production periods.

CHAPTER 4

THE EFFECT OF DIETARY CRUDE PROTEIN ON SEMEN QUALITY AND FERTILITY OF BROILER BREEDER MALES

4.1 INTRODUCTION

The importance of proper management of the body weights of broiler breeders was emphasised in Chapter 2 and some feeding strategies were discussed. It is evident that the growth of broiler breeders needs to be limited in order to maintain or improve fertility. It has been suggested that breeders can be fed lower protein diets as a means of tempering their growth rate with no adverse effects on reproductive performance (Wilson *et al.*, 1987a, Wilson *et al.*, 1987b, Revington *et al.*, 1991, Lopez & Leeson, 1995, Leeson & Summers, 2001). Results from Chapter 3 indicate that a deficiency or excess of dietary protein may impact fertility and that there may be an optimum level of protein associated with maximum fertility.

However, results from experiments that have been conducted on this subject have not been consistent. In some cases, researchers found no significant effect of dietary CP content on semen volume (Wilson *et al.*, 1987a, Wilson *et al.*, 1987b, Wilson *et al.*, 1988, Hocking, 1989, Revington *et al.*, 1991), concentration (Wilson *et al.*, 1987a, Wilson *et al.*, 1987b, Wilson *et al.*, 1988, Revington *et al.*, 1991, Zhang *et al.*, 1999), the metabolic activity of spermatozoa (Hocking, 1989), or fertility (measured as fertile eggs candled at 18 days of incubation per total eggs set) (Fontana *et al.*, 1990, Hocking & Bernard, 1997a). However, others have reported that a high CP diet adversely affected the concentration of spermatozoa (Hocking & Bernard, 1997b, Selvan, 2007), sperm motility (Selvan, 2007), semen volume (Zhang *et al.*, 1999, Selvan, 2007), the percentage of live and normal spermatozoa (Selvan, 2007), the proportion of males producing semen (Wilson *et al.*, 1987a, Wilson *et al.*, 1987b, Hocking, 1989), and fertility (measured as fertile eggs candled at 18 days of incubation per total eggs set) (Hocking, 1990). In another study, a quadratic effect ($P<0.05$) of CP intake on semen volume, sperm concentration, motility, spermatozoa vigour and fertility was observed (Borges *et al.*, 2006b). At extreme levels (deficiency or excess) of protein intake, reproductive performance decreased and the crude fat percentage in the carcass increased (Borges *et al.*, 2006b). An intake of 16.9 g of

CP/b/d was recommended to meet the CP requirements of male broiler breeders from 27 to 61 weeks of age (Borges *et al.*, 2006b).

It has been suggested that any improvements in semen quality and fertility, that could be observed when feeding breeder males diets low in CP, is as a direct consequence of greater control of body weight (Leeson & Summers, 2001). This idea is supported by the findings of Romero-Sanchez *et al*. (2007a) who reported that increasing the dietary CP during rearing (2 to 26 weeks of age) from 120 g CP/kg feed to 170 g CP/kg feed resulted in increased male body weight after four weeks of age. The increased body weight on the high CP diets corresponded to a decline in early and late fertility and it was suggested that the larger body weight males had received inadequate quantities of nutrients during the production period, probably ME, required to support their greater body weight and continued reproductive performance (Romero-Sanchez *et al.*, 2007a).

The process of incorporating feed proteins and amino acids into body proteins is inefficient, and is thus associated with a high heat increment from metabolism (Coon, 2004). Poor quality dietary proteins and amino acids could therefore intensify heat stress conditions in poultry housed in hot climates, resulting in a significant reduction in performance. The nitrogen not used in body gains or production must also be converted into a non-toxic metabolite, uric acid, and eliminated from the body (Coon, 2004). This conversion requires a significant amount of metabolic energy and could thus result in a relative energy deficiency (Hocking, 1989). High plasma concentrations of uric acid may also induce a deficiency in another nutrient or metabolite, and could impair mating efficiency through the deposition of uric acid crystals in the joints of the foot and hock (Hocking, 1989, 1990).

Variations of the protein and amino acid contents of broiler breeder diets are therefore also observed to have an effect on the nitrogen content of the excreta, and this was found to increase with increasing CP contents of the diets (Lopez & Leeson, 1995). This is of vital concern from an economical point of view, since protein is a relatively expensive nutrient in poultry feeds. There are also environmental considerations, as nitrogen losses in animal waste have been shown to have a negative effect on fresh water supply (Coon, 2004).

It is, therefore, evident that there are some discrepancies with regards to the influence of various levels of CP on the reproductive performance of broiler breeder males. The importance of feeding the correct amount of protein to these birds has also been discussed. Therefore, the objective of this experiment was to observe the effect of different levels of dietary inclusion of CP on the semen quality and fertility of breeder males, while controlling body weight and keeping energy levels of the feed constant.

4.2 MATERIALS AND METHODS

Fifty two Ross 788 broiler breeder males were obtained from the Ross breeder house at the poultry section on Ukulinga Research Farm where they had been housed together with broiler breeder females of the same strain and reared according to the breeder recommendations. The males were removed from the breeder flock at 22 weeks of age and were then housed in individual cages that were raised above the floor. There was a nipple drinker inside each cage and a feed basin was attached to the outside of each cage. The males were provided with *ad libitum* access to water throughout the experimental period. The breeder males were fed a commercial broiler breeder pellet (140 g/kg CP, 11.5 MJ/kg AME) up to 26 weeks of age.

After caging, the males were handled (trained) once weekly in order to stimulate erectile and ejaculatory responses to the abdominal massage method (Burrows & Quinn, 1937). Records were kept of individual responses to each training session. After four weeks of training, 36 males, that were observed to respond positively to massage, were selected and then randomly allocated to their respective experimental diets. The remaining males were culled.

Two basal diets (a summit and a dilution diet) were formulated. The summit diet was formulated according to the breeder nutrient specifications of male feed from 24 weeks onwards (Arbor-Acres-Plus-Aviagen, 2005). The dilution diet was formulated according to the same nutrient levels, but the objective function was to “minimise total essential amino acids” and ingredients with almost negligible amounts of protein contents were used. In doing this, a dilution diet was created that contained very little crude protein but similar amounts of all other nutrients as the summit diet. The ingredient contents of the two basal diets are given in Table 4.1.

Table 4.1 *Dietary ingredient contents (g/kg) for the two basal diets used in the experiment*

Ingredient	Summit	Dilution
Yellow maize fine	619.81	
Starch		605.08
Sand		202.41
Wheat bran	188.70	
Soybean 50	88.04	
Sunflower 37	47.38	
Sunflower husks	26.37	116.33
Sunflower oil		28.75
Limestone	16.73	14.64
Monocalcium phosphate	6.78	14.00
Potassium carbonate		10.52
Sodium bicarbonate	2.82	2.70
Choline chloride 60%		1.92
Vitamin & mineral premix	1.50	1.50
Salt	1.49	2.14
L-lysine HCl	0.26	
DL methionine	0.09	
L-threonine	0.03	

The nutrient compositions of the two basal diets were calculated in order to determine the ratio in which to blend them to get the required crude protein levels in the experimental diets and to ensure that they had the same energy content. The two basal diets were mixed and then blended together in various proportions to form the three experimental diets (Table 4.2).

Table 4.2 *The percentage compositions of the three experimental diets*

Experimental diet	Protein level	% Summit	% Dilution
1	Low	65	35
2	Control	82	18
3	High	96	4

After blending the two basal diets, a sample of each experimental diet was analysed for their respective nutrient compositions (Table 4.3) according to the methods described in Appendix D.

Table 4.3 *The calculated and analysed nutrient contents (units specified) of the three experimental diets*

Nutrient	Unit	Diet 1		Diet 2		Diet 3	
		Calculated	Actual	Calculated	Actual	Calculated	Actual
AMEn_adult	MJ/kg	11.09	10.41	11.09	10.39	11.09	9.97
EE	MJ/kg	9.82		9.80		9.79	
Crude protein	%	9.70	10.46	12.03	12.61	13.95	15.00
Dry matter	%	90.47	88.60	88.68	86.70	87.20	87.40
Lysine	%	0.37	0.30	0.46	0.43	0.53	0.51
Methionine	%	0.16		0.20		0.23	
Cysteine	%	0.16		0.20		0.23	
Methionine + cystine	%	0.31	0.09	0.40	0.19	0.46	0.24
Threonine	%	0.30	0.18	0.37	0.30	0.43	0.45
Tryptophan	%	0.09		0.11		0.13	
Arginine	%	0.54	0.42	0.67	0.38	0.78	0.81
Isoleucine	%	0.33	0.28	0.41	0.39	0.48	0.51
Leucine	%	0.81	0.64	1.01	0.82	1.18	1.13
Histidine	%	0.25	0.30	0.31	0.40	0.36	0.40
Phenylalanine	%	0.38	0.29	0.47	0.46	0.55	0.53
Tyrosine	%	0.29	0.22	0.36	0.28	0.42	0.34
Phenylalanine + tyrosine	%	0.67	0.51	0.83	0.74	0.97	0.87
Valine	%	0.42	0.36	0.52	0.42	0.61	0.69
Ash	%	3.84		4.09		4.30	
Crude fibre	%	5.70		5.70		5.70	
Crude fat	%	3.43		3.43		3.43	
Calcium	%	0.80	0.82	0.80	0.83	0.80	1.11
Available phosphorous	%	0.35	0.55	0.35	0.61	0.35	0.61
Sodium	%	0.16		0.16		0.16	
Chloride	%	0.16		0.16		0.16	
Potassium	%	0.70		0.70		0.70	
Linoleic acid	%	1.81		1.79		1.78	
Choline	mg/kg	1000.00		1000.00		1000.00	
Selenium	ng/g		210.80		223.47		242.64

Feed allocation was calculated on a weekly basis, based on the mean body weight of all the birds, to adhere to the breeders' recommended growth curve for male parent stock (Arbor-Acres-Plus-Aviagen, 2005). This was done in order to avoid confounding of BW and CP treatment effects. The feed was weighed out and the birds were fed in the mornings. The males were fed the experimental diets from 26 to 41 weeks of age.

The broiler breeder males were weighed individually at the beginning of the trial and once a week thereafter. An electronic scale was used and the males were placed head-first inside a cone during weighing in order to restrain them and to avoid injuries.

After the males had been on experimental diets for a period of a week, semen was used for artificial insemination of commercial layer hens. This was done in order to assess the ability of spermatozoa to survive in the hen (Wishart, 1987, Brillard & Bakst, 1990), and to possibly predict the fertilising ability of semen samples (Brillard & Antoine, 1990, Wishart, 1997).

The feathers around the cloaca of each male were clipped in order to minimise contamination of the semen during collection. Care was taken to avoid collecting the transparent watery fluid that is characteristically produced in the fowl (Lake & Stewart, 1978) at ejaculation in response to massage. If it is collected in abundance, it serves to dilute semen reducing the number of spermatozoa contained in a unit volume. The semen was collected into small plastic flip top vials. Semen from each male was diluted in a 1:1 ratio with Tyrodes before collection from the next male. Semen was collected from six males of each CP treatment group. Immediately after all of the semen samples had been collected and diluted, laying hens were inseminated with 0.4 ml of diluted semen (i.e. 0.2 ml semen and 0.2 ml diluent) with an Eppendorf pipette. The semen was released slowly into the hen so as to stop semen running out of the vagina. A new pipette tip was used for the semen from each male in order to prevent any contamination of the semen samples. Artificial insemination was performed in the late afternoon, when most of the laying hens had already laid their egg for the day (Donoghue *et al.*, 1995, Brillard, 2003). The insemination was performed once every two weeks, except for a four week period (males aged 32 to 36 weeks), for the entire duration of the trial.

It was decided that the hens would be inseminated with a constant volume of semen rather than a constant number of spermatozoa. It would take a considerable amount of time to calculate the semen concentration prior to diluting it in the correct ratio in order to get a certain number of spermatozoa per insemination dose. The amount of time between collecting of semen, dilution and insemination needs to be kept to a minimum as the spermatozoa are highly active and would thus produce waste products that would impair their normal function or even kill them after a short period of time (Etches, 1996). Since

there was no trained staff that could determine the semen concentration while the semen samples were being collected from the remaining males, it was decided that this would not be done.

A larger than usual volume of semen was used for each insemination so as to ensure complete filling of the SST in the hen (Brillard & McDaniel, 1986, McDaniel *et al.*, 1997b). This would enable the determining of the fertilising potential of the males without needing to measure concentration, since only metabolically active spermatozoa with intact plasma membranes are stored in the SST (Bakst *et al.*, 1994). It could also reduce the effect of variation between different females to some extent (Wishart & Palmer, 1986). Laying hens, instead of breeder females, were also used in order to reduce the female effect on fertility as they have been found to have significantly ($P<0.05$) more SST with a higher percentage of stored spermatozoa than broiler strain hens (Van Krey *et al.*, 1971).

The day on which insemination was performed was referred to as Day 0 (D0). Eggs were collected on D2, D3, D4, D6, D7, D8 and D14 from all the inseminated hens and stored in a cold room for no more than two weeks before being assessed.

The eggs were brought to room temperature, cracked open, and the yolks were separated from the albumen. Excess albumen was removed by rolling the yolk on a paper towel. A square of approximately 1 x 1 cm of the perivitelline layer situated around the germinal disk area was then cut and removed. This piece of membrane was rinsed in PBS to remove adherent yolk, before being stretched out on a glass microscope slide. It was then stained with a 1 μg/ml solution of DAPI in PBS and covered with a cover slip (Wishart, 1987). The slides were placed in a light tight container immediately after they were stained and were examined by fluorescence microscopy within three hours.

The sperm nuclei embedded in the outer perivitelline layer in 20 randomly-chosen fields of view (each 177687.19 μm^2) were counted and totalled (Figure 4.1a). The sperm nuclei were characteristically comma-shaped in appearance, but occasionally some were seen in a coiled configuration (Figure 4.1b). The numbers of sperm nuclei were calculated as sperm per mm^2 of membrane.

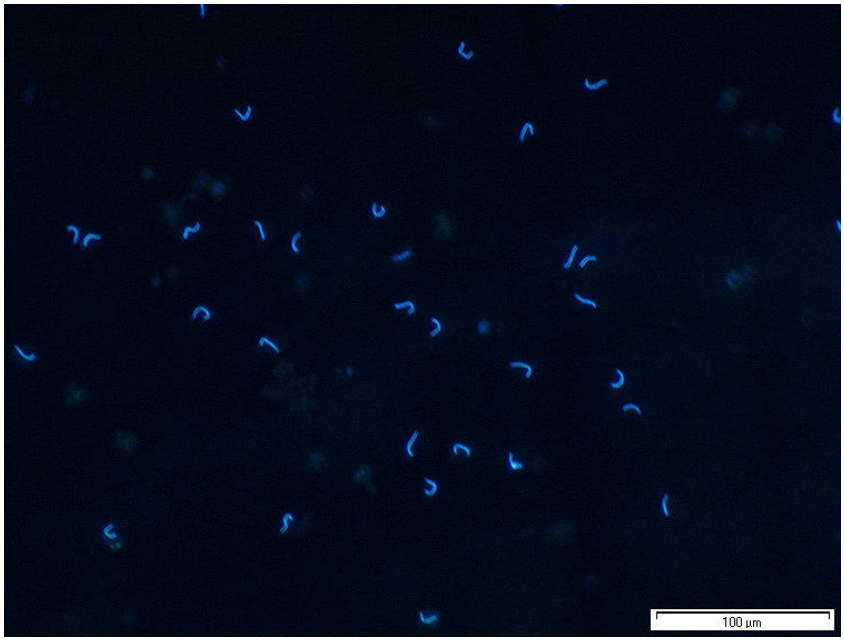

Figure 4.1a *Fluorescent nuclei of spermatozoa trapped in the outer perivitelline membrane of an oviposited egg; visible under fluorescence microscopy after staining with a 1 µg/ml solution of DAPI in PBS. Scale bar represents 100 µm.*

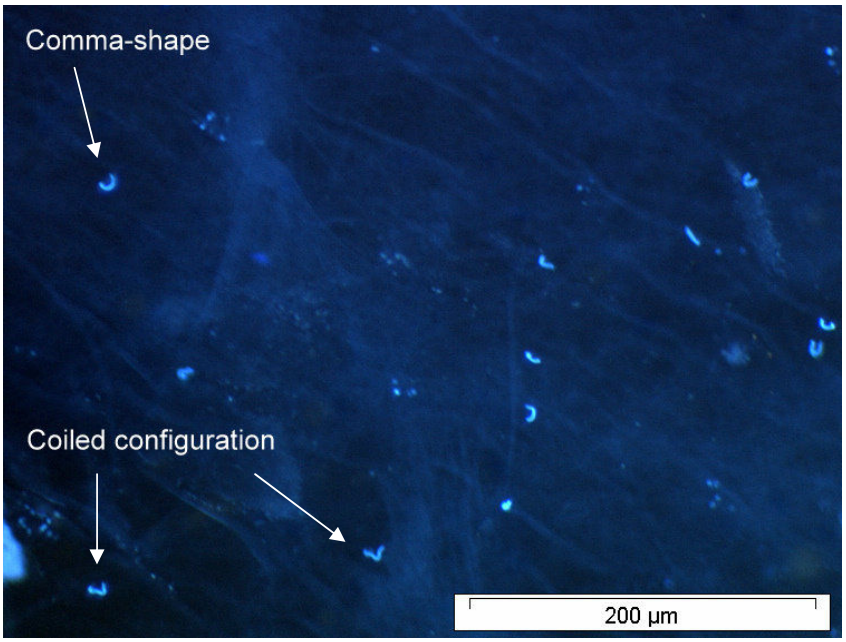

Figure 4.1b *Fluorescent nuclei of spermatozoa trapped in the outer perivitelline membrane of an oviposited egg; visible under fluorescence microscopy after staining with a 1 µg/ml solution of DAPI in PBS. Scale bar represents 200 µm.*

The fertility of the eggs was predicted according to two methods: one which requires more than three $OPVL_{sperm}$ per mm^2 for an egg to be considered fertile (Wishart, 1997) and another which requires 0.43 $OPVL_{sperm}$ per mm^2 for an egg to be considered fertile (Brillard & Antoine, 1990).

Semen was collected from six males per treatment, once every two weeks for the purpose of artificial insemination. In order to examine some semen quality traits, semen was collected on the following day from the 18 remaining males. Thus each male represented a replicate of its respective treatment, however, not every male yielded semen on all of the collection days. On every alternate week, the males were massaged and their response was recorded, but no semen was collected. This was done in order to maintain semen production (Riaz *et al.*, 2004).

- **Morphology**

Immediately after semen collection, a drop of undiluted semen was placed in 100 µl of 3 percent glutaraldehyde and mixed lightly. This is done in order to fix and preserve the spermatozoa in their state at ejaculation. These samples were then kept to be examined at a later date. A drop of the sample was placed onto a microscope slide and gently covered with a cover slip before being examined with a light microscope at 40 times magnification. Three hundred spermatozoa on each slide were counted at random and defects were noted and then calculated as a percentage of the total observed. The following defects could be noted: sperm bent at head-midpiece or midpiece-tail junctions, knots or swelling at head-midpiece junctions or in the head region, and coiled heads (Lake & Stewart, 1978, Alkan *et al.*, 2002).

- **Concentration**

Prior to semen collection, solutions containing Eosin and Nigrosin were prepared for each semen sample. A known volume of undiluted semen (either 50 or 100 µl, depending on the volume of semen yielded by the bird) was placed within the Eosin and Nigrosin solution and mixed well by rotating the bottles gently. A small amount of the mixture was then used to fill the two sides of a haemocytometer. Spermatozoa were counted in 10 random blocks on either side of the haemocytometer at 40 times magnification using a light microscope.

This data, together with the corresponding mass motility scores, were then used in a formula (Appendix C) to calculate the number of live sperm per ml of semen.

- **Motility**

After removing the semen needed for the concentration and morphology assessments, the remainder of each semen sample was immediately diluted with Tyrodes solution and placed aside before proceeding to the next male. Once semen had been collected from all of the males, the motility assessment was done. A drop of the diluted semen sample was placed on a pre-warmed slide and gently covered with a cover slip. A light microscope at 40 times magnification was used to examine the sample. Three random areas on the slide were examined in order to score the percentage progressive motile normal sperm, the percentage abnormal motile sperm and the percentage dead sperm. Care was taken to minimise the time between collecting and analysing the semen motility.

Statistical Analysis

The frequency of eggs considered fertile per male CP treatment according to the predictions of Wishart (1997) (W) and of Brillard & Antoine (1990) (BA) were subjected to a Chi-Square test of independence using *Minitab* (Minitab-Inc., 2007). This was done in order to test the null hypothesis that fertility and CP treatments are not associated.

Unless stated otherwise, *GenStat 11th Edition* (Lawes-Agricultural-Trust, 2008) was used for all further data analyses as well as any graphical output.

Binomial logistic regression was used to determine the effects of male age, days post-insemination and crude protein on fertility, as predicted by W. The fertility data (1 = fertile, 0 = infertile) were transformed to normal equivalent deviates (probits) and the factors were fitted to the model. The reference levels for the factors were: 2, 27 and 104.6 for day post-insemination, male age (weeks), and male treatment (g CP/kg feed), respectively.

The numbers of $OPVL_{sperm}$ observed in all of the eggs collected from laying hens on D2, D3, D4, D6, D7, D8 and D14 post-insemination were subjected to a general ANOVA with an unbalanced treatment structure. This type of ANOVA was used as an unequal number of eggs represented each CP treatment group. Significant effects of age ($P<0.001$) and day post-insemination ($P<0.001$) on the number of $OPVL_{sperm}$ were observed, and thus these were blocked as "nuisance terms" in the ANOVA model in order to focus on the CP treatment effect. The $OPVL_{sperm}$ means were compared using the appropriate LSD values at 5 percent level of significance. Exponential curves were fitted with standard curve regression to observe the relationship between $OPVL_{sperm}$ and day post-insemination or male age.

The percentage fertility of eggs obtained from laying hens was predicted from the median $OPVL_{sperm}$ per mm^2 values of each group at different days post-insemination (Staines *et al.*, 1998). The calculated percentage fertility data were subjected to a general ANOVA and differences in the overall CP treatment means were compared by the LSD value at 5 percent level of significance. Exponential curves were fitted with standard curve regression to observe the relationship between the predicted percentage fertility and male age.

The semen morphology, concentration, motility and number of live sperm per ml of semen data were subjected to a general ANOVA. The treatment structure was unbalanced due to the different number of birds from each treatment that yielded semen on the collection days. All means were compared using the appropriate LSD values at 5 percent level of significance. Exponential curves were fitted with standard curve regression to observe the relationship between percentage normal morphology and age for the CP groups.

One male died due to unnatural causes (put its neck out of the cage and back into the cage through a different gap, the comb got stuck and prevented it from pulling the head back out, thus the bird probably suffocated) and two males went missing during the trial period. The mortality records were not analysed as the deaths were not related to CP treatments.

The body weights of the males at the beginning of the experiment were subjected to a general ANOVA. The body weights of the remainder of the experiment were subjected to a general ANOVA with an unbalanced treatment structure. This type of ANOVA was used since there were an unequal number of males in each CP treatment group, due to the reasons explained above. Mean comparisons of BW from birds of different CP treatments were made using the appropriate LSD value at 5 percent level of significance. Exponential curves were fitted with standard curve regression to observe the relationship between body weight and male age.

The total counts of positive and negative responses of the males on each CP treatment to the abdominal massage method, during the course of this trial, were subjected to a Chi-Square test of independence using *Minitab* (Minitab-Inc., 2007). This was done in order to test the null hypothesis that semen yield and CP treatments were not associated.

4.3 RESULTS AND DISCUSSION

The Chi-Square values obtained from the counts of fertile and non-fertile eggs were as follows: 4.117 ($P = 0.128$) and 1.529 ($P = 0.465$), for W and BA respectively. The null hypothesis was thus accepted in both cases and no further tests were done between the CP treatments.

Two of the three factors fitted to the logistic regression model were significant ($P<0.001$), but there were no significant interactions between any of the factors. The estimates of W on D4, D6, D7, D8 and D14 differed significantly ($P<0.05$) from D2. Fertility estimates of W at 27 weeks differed ($P<0.05$) to those at 31, 37 and 41 weeks. There were no differences in the estimated values of W between the low and other CP groups, although the control CP group was approaching a level of significance ($P = 0.063$). The estimates of all of the significant factors were negative; suggesting that W decreases with an increase in male age and day post-insemination.

A significant difference ($P<0.05$) was found between the mean number of $OPVL_{sperm}$ observed in eggs collected from laying hens when semen of males of different dietary CP treatments were used for the insemination. Semen of the control CP group yielded eggs

with a significantly higher mean $OPVL_{sperm}$ per mm^2 than that of the low and high CP groups (Figure 4.2). There was no significant difference between the low and high CP treatment groups. The higher number of $OPVL_{sperm}$ observed in the control CP group than in the high CP group is different to what Hocking & Bernard (1997a) found. They observed no effect of protein content (120 versus 160 g CP/kg feed) on the number of sperm trapped in the PVL of eggs collected from naturally mated breeders. It is possible that a level of 120 g CP/kg feed may have already been limiting sperm production or functioning to some extent, and thus no benefit, with regards to PVL sperm numbers, were observed when lowering the CP content of the feed.

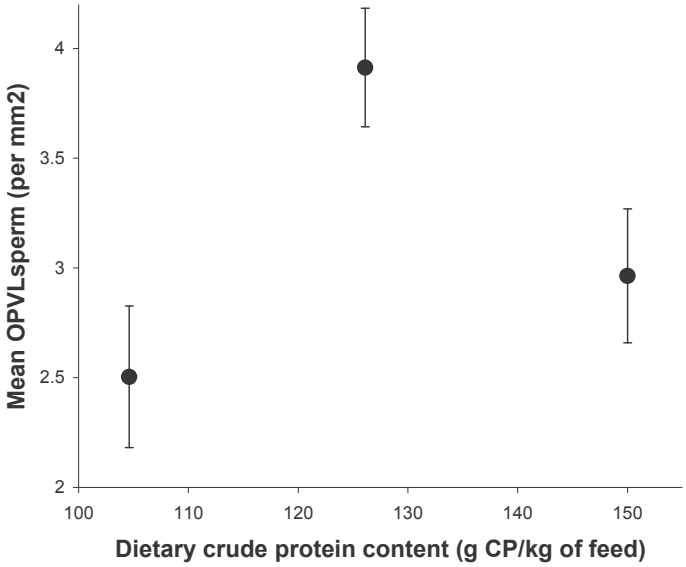

Figure 4.2 *Means of all $OPVL_{sperm}$ per mm^2 values of eggs obtained from laying hens after insemination from males fed different dietary levels of crude protein.*

There was a significant ($P<0.001$) effect of day post-insemination on the number of $OPVL_{sperm}$ within oviposited eggs across all levels of CP. Separate exponential curves ($P<0.001$; estimated S.E. = 3.53, 4.78, and 3.16, for low, control and high CP groups respectively) were fitted to the treatment groups (Figure 4.3). The relationship was similar to that observed by Wishart (1987). Eggs on D2 contained a significantly higher amount of $OPVL_{sperm}$ than any of the other days post-insemination. There were no significant differences between eggs collected on D4, D6, D7, and D8 post-insemination. Eggs collected on these days had significantly fewer $OPVL_{sperm}$ than those collected on D3, but

had significantly higher amounts of $OPVL_{sperm}$ than eggs of D14. There was a large amount of variation within the treatment groups with regards to the number of $OPVL_{sperm}$ on each day.

The y-axis scale of Figure 4.3 was narrowed in order to make the individual points more visible (Figure 4.3b). It is evident that the eggs that were inseminated with semen of the control CP group had a higher content of $OPVL_{sperm}$ than those of the low and high CP groups, throughout the 14 day collection period. Because a chicken egg can be considered fertile, with 94 percent certainty, if more than three $OPVL_{sperm}$ per mm^2 are found within the egg (Wishart, 1997), a straight line (y = 3) was fitted to the graph in Figure 4.3b to depict the days post-insemination that eggs become infertile. The intercept of each curve with this line represents the day post AI at which eggs becomes infertile. These values were 3.20, 5.11 and 4.25 days in eggs inseminated with semen from the low, control and high CP groups, respectively, showing a longer duration of fertility of eggs when semen from the control males was used for insemination. It is important to note, however, that no data were collected from eggs on D9 to D13 post-insemination. However, because fertility had declined by D8, it was not considered necessary to assess the $OPVL_{sperm}$ contents of eggs of D9 to D13 post-insemination.

The age of the males also had a significant ($P<0.001$) effect on the mean number of $OPVL_{sperm}$ in oviposited eggs. Separate exponential curves ($P<0.001$; estimated S.E. = 3.92, 5.17, and 3.63, for low, control and high CP groups respectively) were fitted to the treatment groups (Figure 4.4). The fitted curves had very low R^2-values as there was a large amount of variation within the CP groups with regards to the number of $OPVL_{sperm}$ at each age. A decrease in the number of $OPVL_{sperm}$, with increasing age, was observed regardless of the CP treatment. The number of $OPVL_{sperm}$ was greater in the control CP group than in the other groups at all of the observed ages except at 29 and 31 weeks, where a single observation was higher in the low and high CP groups, respectively. Hocking & Bernard (1997a) also found a significant ($P<0.05$) relationship between the number of sperm trapped in the PVL and bird age, however, unlike the current trial, an increase in trapped sperm with increasing age was observed in eggs assessed at 28, 38, and 48 weeks of age.

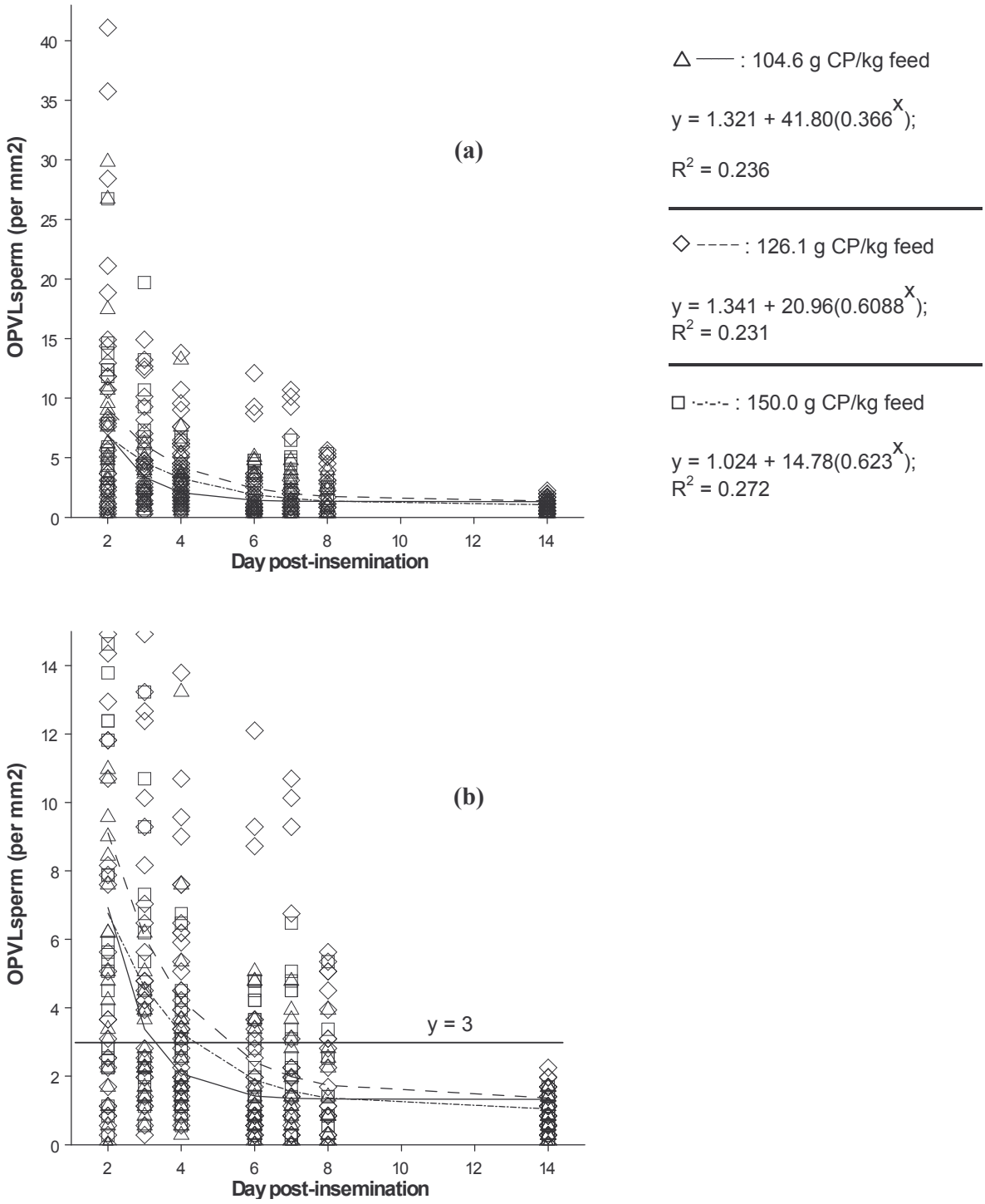

Figure 4.3 *Number of $OPVL_{sperm}$ per mm^2 of eggs obtained from laying hens on D2, D3, D4, D6, D7, D8 and D14 post-insemination.*

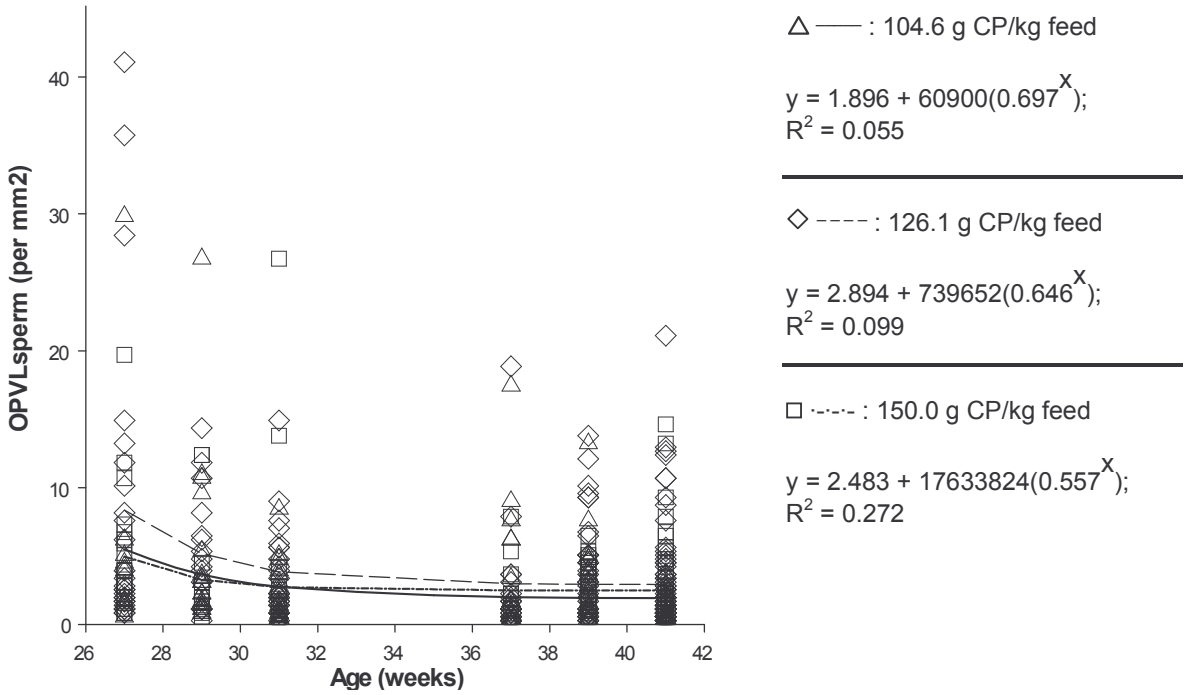

Figure 4.4 *Number of $OPVL_{sperm}$ per mm^2 of eggs obtained from laying hens after insemination from males of different ages.*

Staines *et al.* (1998) found a significant correlation (r = 0.766) between flock fertility and the median $OPVL_{sperm}$ (per mm^2) from samples of approximately 60 eggs from each of 19 different broiler breeder flocks aged between 35 and 70 weeks: percent fertility = 59 + 2.80 x median $OPVL_{sperm}$ per mm^2. This relationship was used to predict the percent fertility of the eggs collected in the current experiment, at different days post-insemination (Figure 4.5). Separate exponential curves ($P<0.001$; estimated S.E. = 0.52, 1.73, and 1.54, for low, control and high CP groups respectively) were fitted to each CP treatment group. The median divides a sample into two equally sized groups, and its value will therefore depend on the distribution of the sample data. The distributions of the $OPVL_{sperm}$ data were positively skewed, the degree of skewness being inversely proportional to the amount of CP in the treatment feeds, i.e. high > control > low CP. These predicted fertility values are thus only applicable to this experiment, and should not be compared with the fertility of other breeder flocks or experiments.

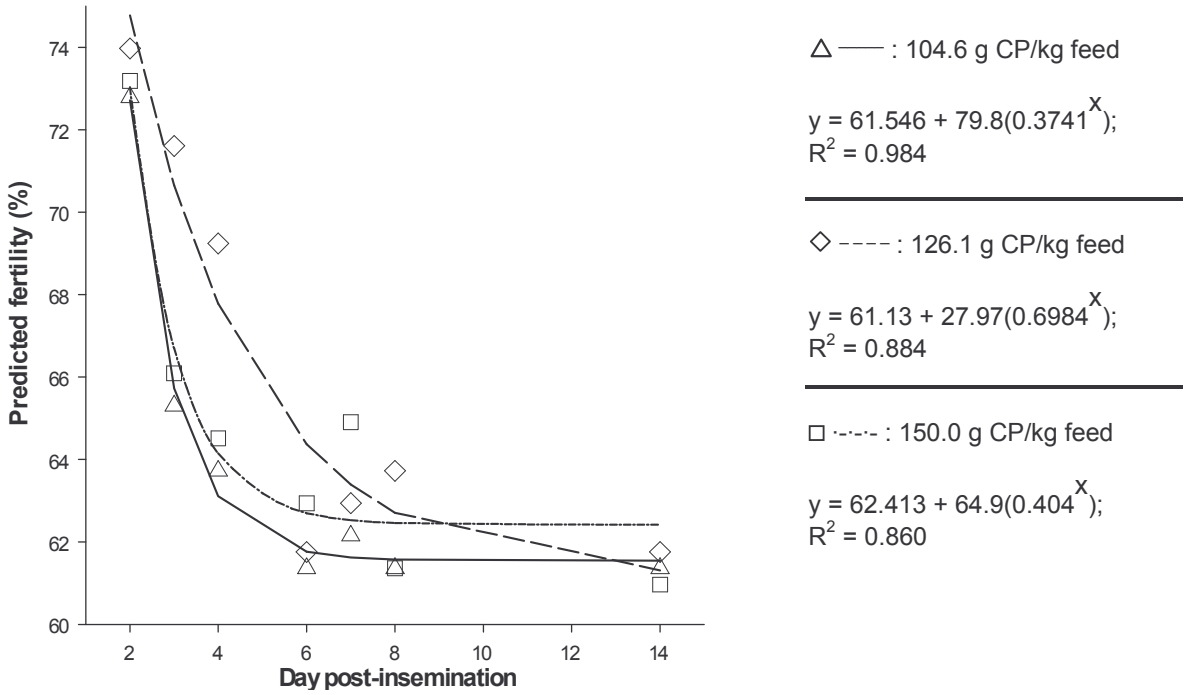

Figure 4.5 *Predicted percentage fertility based on a relationship found between fertility and the median frequency (Staines et al., 1998) of* $OPVL_{sperm}$ *per* mm^2 *of successive eggs obtained from laying hens after artificial insemination.*

The shapes of the curves fitted to the data of Figure 4.5 are similar to those fitted to Figure 4.3. This could be expected, since the median values used in the equation of Staines *et al.* (1998) were derived from the $OPVL_{sperm}$ data that was plotted in Figure 4.3. When this equation is used though, fertility is above 59 percent, even at 14 days post-insemination, which may not necessarily be correct. No significant effect of CP treatment was found on the predicted percent fertility, but it appears that the control CP group had higher percentage fertility, on most of the post-insemination days, than the other treatment groups.

Other researchers also observed no significant difference in fertility (measured as fertile eggs candled at 18 days of incubation per total eggs set) between breeder males fed diets containing 120 g/kg CP and either 140 g/kg CP (Fontana *et al.*, 1990) or 160 g/kg CP (Hocking & Bernard, 1997a). Hocking (1990) found that naturally mating males on a 110 g/kg CP diet yielded eggs with a significantly ($P<0.05$) higher fertility (measured as fertile eggs candled at 18 days of incubation per total eggs set), than males fed 160 g/kg CP diets. However, this difference was only significant during the latter part of the breeding period

(42 to 60 weeks of age). Since the current study was ended at 42 weeks of age, it is not possible to predict what would have happened beyond that age. However, the findings of Hocking (1990) suggests that as the birds age, their CP requirements become lower. This possibility, together with the higher $OPVL_{sperm}$ numbers observed in eggs from the control CP group than in the high CP group of the current experiment, may suggest that fertility would have been higher in the control CP group than in the high CP group.

No significant differences were observed in the semen morphology of males of the different CP treatment groups (Table 4.4). The most prevalent morphological defects observed in this study were sperm that were bent or that had a knot at the head-midpiece junction. This finding is similar to that of the previous trial (Chapter 3) and confirms the suggestion that the midpiece of poultry semen deteriorates quicker than other regions (Lake & Stewart, 1978, Maeda *et al.*, 1986). There was a significant exponential ($P<0.001$; estimated S.E. = 3.88, 2.17, and 2.36, for low, control and high CP groups respectively) effect of age on the morphology of the semen samples. The percentage of normal spermatozoa decreased with increasing age in all of the CP treatment groups (Figure 4.6). Even though there were no significant differences between the treatment groups in the overall morphology of their respective semen samples, it is evident that the low CP group had a much lower percentage of normal spermatozoa at 39 weeks of age. The amount of variance accounted for by the fitted exponential curves of each treatment group was relatively low, particularly that of the control CP group. This was due to a large variation between observations within each CP treatment group.

Table 4.4 *Mean values of morphology (± s.e.m.) of semen obtained from Ross broiler breeder males fed different dietary levels of crude protein from 26 to 41 weeks of age*

Treatment (CP level)	Morphology (%)						
	Normal	Bent	Coiled heads	Knots in head	Knots at head-midpiece junction	Swelling in head	Swelling at head-midpiece junction
Low	95.0 ± 0.7	2.6 ± 0.5	0.3 ± 0.1	0.2 ± 0.1	1.0 ± 0.2	0.3 ± 0.1	0.7 ± 0.2
Control	94.8 ± 0.6	3.2 ± 0.4	0.2 ± 0.1	0.2 ± 0.1	0.9 ± 0.2	0.3 ± 0.1	0.5 ± 0.2
High	94.9 ± 0.7	2.3 ± 0.5	0.2 ± 0.1	0.2 ± 0.1	1.0 ± 0.2	0.4 ± 0.1	1.1 ± 0.2

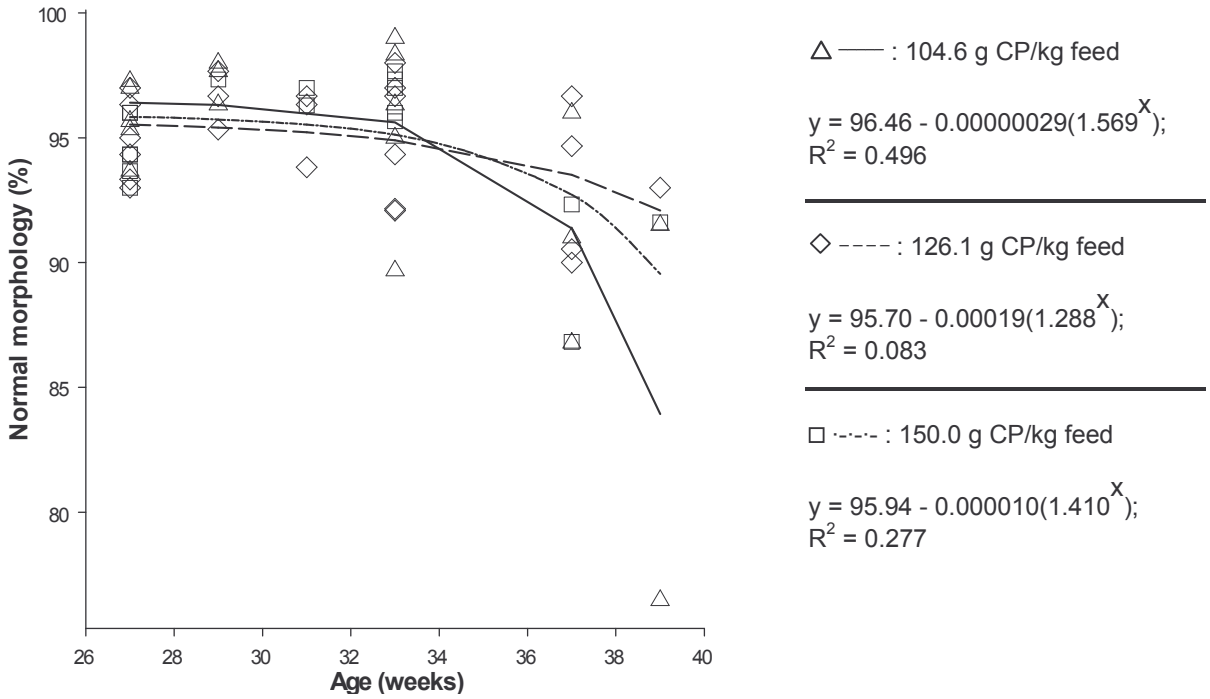

Figure 4.6 *Percentage of normal morphology observed in semen samples of progressively ageing males fed different levels of dietary crude protein.*

Semen of the high CP group had a significantly ($P<0.05$) lower percentage of progressive normal motile spermatozoa than semen of the low or control CP groups (Table 4.5). There were no significant differences in the percentage of abnormal motile or non-motile sperm motility in males on different CP diets. The high CP group had a higher semen concentration and number of live sperm per ml of semen sample than the other treatment groups, although the difference was not significant. This is different to the observations of Hocking & Bernard (1997b) who found significantly ($P<0.05$) lower semen concentrations in males on a high CP diet (160 g CP/kg) than males on a lower CP diet (120 g CP/kg). At the end of their trial, a large proportion of males on the high CP diet had small, non-functioning testes.

Sperm motility was only assessed once semen was collected from all of the males. The observed higher motility in semen of the low and control CP groups, in the current study, may therefore be attributed to their observed lower semen concentration. It is possible that the functioning of spermatozoa in the semen samples of the high CP group may have deteriorated at a faster rate than that of the other groups as there was a higher concentration

of sperm cells that were producing waste products. It is important to note, however, that under natural mating conditions, a higher semen concentration would not adversely affect sperm motility, as the seminal fluid of the male and the SST of the female would provide an environment that would preserve the functioning of normal spermatozoa (Bakst *et al.*, 1994).

Table 4.5 *Mean values (± s.e.m.) of motility, semen concentration and numbers of live sperm per ml of semen obtained from Ross broiler breeder males fed different dietary levels of crude protein from 26 to 41 weeks of age*

Treatment (CP level)	Motility (%)			Concentration sperm/ ml ($x10^6$)	Number of live sperm/ ml ($x10^6$)
	Progressive normal motile	Abnormal motile	Non-motile (dead)		
Low	74.4[a] ± 2.7	10.6 ± 1.1	15.1 ± 2.4	96.0 ± 18.2	84.0 ± 16.6
Control	81.5[a] ± 2.3	8.8 ± 0.9	9.8 ± 2.1	93.9 ± 16.1	84.5 ± 14.7
High	72.8[b] ± 2.6	11.7 ± 1.1	15.5 ± 2.3	121.7 ± 17.7	101.3 ± 16.1

[a,b] Means in a column followed by different superscripts are significantly different at 5 percent level of significance

Even though the all of the males were randomly allocated to the CP treatment groups at 26 weeks of age, there was a non-significant (P = 0.444) difference in the mean BW of each CP treatment group at the beginning of the experiment. At 26 weeks of age, males in the low, control and high CP group had mean body weights of 4.143, 4.188 and 4.304 kg, respectively. All of the males received the same amount of feed, with almost the same levels of energy, yet a difference in BW could be observed throughout the duration of the experiment (Figure 4.7). A significant (P<0.001) difference was observed between the overall mean body weights of males of the all of the CP treatment groups. Exponential curves (P<0.001; estimated S.E. = 0.272, 0.307, and 0.260, for low, control and high CP groups respectively) were fitted to the body weights of the CP groups. The R^2 – values of the curves fitted to each of the CP treatment groups were low, indicating that there was a large variation between the males within each group. Males fed the low or control CP diets had lower body weights than the high CP males throughout the trial period.

It is possible that the difference in BW throughout the trial may be attributed to a difference in the initial body weight means of the CP groups. However, the difference at the beginning of the trial was not significant, and a lot of variation between males in each

treatment group was observed throughout the experiment (Figure 4.7). Also, Wilson *et al.* (1987b) observed increased body weights, with an increase in dietary CP level, in broiler breeder males fed 90, 120 or 150 g/kg CP diets (isoenergetic) on a restricted basis until the birds were 50 weeks of age. It is, therefore, possible that the birds in the higher CP group may have converted the extra protein into muscle tissue, resulting in a higher overall mean body weight.

At 34 weeks of age the mean BW of all of the males was 291 g above the target BW. The feed allocation was therefore decreased by 2 g/b/d, resulting in a mean BW loss of 234 g during the following week. Due to the large loss in mean BW, the feed allocation was increased by 1 g/b/d, preventing further BW losses.

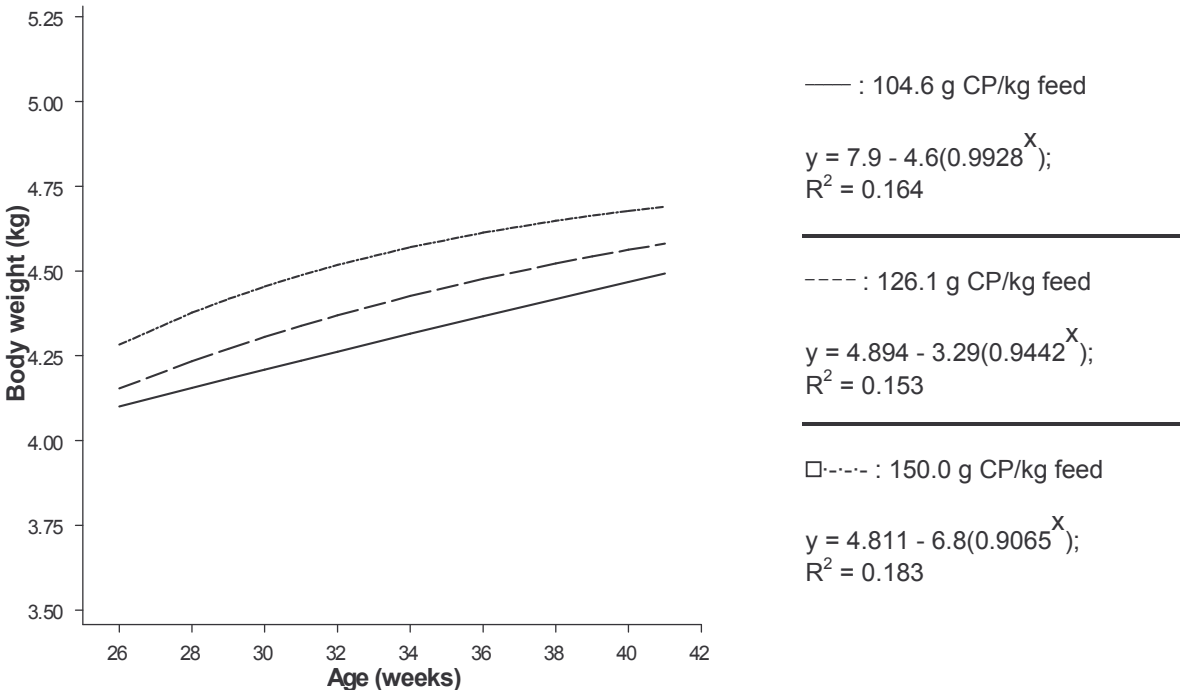

Figure 4.7 *Body weight of Ross broiler breeder males fed different dietary levels of crude protein from 26 to 41 weeks of age.*

The Chi-Square value obtained from the total counts of positive and negative responses of the males in each CP treatment group to semen collection, during the entire duration of the trial, was as follows: 5.847 (P = 0.054). The null hypothesis was thus accepted and no further tests were done between the CP treatments with regards to the frequency of semen

yielded. It is important to note, however, that the P-value was approaching a level of significance.

The mean proportion of males of each treatment group that yielded semen in response to abdominal massage during the trial period is shown in Table 4.6. It is evident that there was a lower mean percentage of males that produced semen in the low and high CP treatment groups than in the control group. These findings are similar to those of other researchers who found that the proportion of males producing semen declined with increasing dietary CP contents (Wilson *et al.*, 1987a, Wilson *et al.*, 1987b, Hocking, 1989). However, Zhang *et al*. (1999) observed no difference between CP diets (120 versus 160 g CP/kg feed) with regards to the proportion of males in semen production.

At 29 weeks of age, there was a lower proportion of males of all of the treatment groups that yielded semen. The feed allocation of the males was decreased at 28 weeks of age, and thus the birds may have been under a higher than usual amount of stress while adapting to a lower amount of feed, as well as a different form of feed (i.e. mash versus pellets). An external factor, such as an extreme in ambient temperature, may also have contributed to the observed decrease in response to abdominal massage.

Table 4.6 *Mean percentage (± s.e.m.) of males that produced semen in response to abdominal massage at various ages during the trial period*

Age (weeks)	Males producing semen (%)		
	Low CP	Control CP	High CP
27	91.7 ± 8.3	100	100
29	75.0 ± 13.1	75.0 ± 13.1	50.0 ± 15.1
30	91.7 ± 8.3	91.7 ± 8.3	83.3 ± 11.2
31	83.3 ± 11.2	91.7 ± 8.3	91.7 ± 8.3
37	75.0 ± 13.1	91.7 ± 8.3	83.3 ± 11.2
39	66.7 ± 14.2	91.7 ± 8.3	66.7 ± 14.2
40	91.7 ± 8.3	100	66.7 ± 14.2
41	90.0 ± 10.0	100	100
Mean over entire period	83.0 ± 3.6	92.6 ± 3.6	80.2 ± 3.6

The current level of crude protein recommended by the breeder for broiler breeder males aged 21 weeks onwards is 120 to 140 g CP/kg feed (Aviagen, 2007). It is also recommended that the females be fed diets ontaining 145 to 155 g CP/kg feed during the

breeding stage (Aviagen, 2007). Therefore, if the males have access to the female feed, either because of an ineffectively designed separate-sex feeder, or due to no separate-sex feeding system being in place, the males will be eating feed with a higher than recommended level of dietary CP. This could potentially have negative effects on broiler breeder male fertility.

4.4 CONCLUSIONS

After inseminating laying hens with a fixed volume of semen of males on different dietary CP treatments, semen of the control CP group yielded eggs with a significantly ($P<0.05$) higher mean $OPVL_{sperm}$ per mm^2 than the other CP groups. This was the common trend on different days post-insemination and at different male ages. The duration of fertility also appeared to be longer in the control CP group than in the low or high CP groups. Crude protein treatment had no significant effect on semen morphology, concentration or the number of live sperm per mm^2. Semen of males of the high CP group had a significantly ($P<0.05$) lower percentage of normal motile spermatozoa than semen of males of the low or control CP group. Although there was no significant treatment effect on the number of males that yielded semen in response to abdominal massage, the P-value was approaching a level of significance ($P = 0.054$). It appeared that a lower percentage of males of the low and high CP groups produced semen in response to abdominal massage than the control CP group.

A significant ($P<0.001$) difference was observed between the overall mean body weights of males fed equal amounts of feed, with similar energy contents, but different CP contents. Males fed the low or control CP diets had lower BW means than the high CP males throughout the trial period. This result, together with the fertility related findings mentioned above, may support the suggestion that broiler breeder males could be fed lower protein diets than what is recommended for breeder females. Provided that they are given adequate CP in order to meet their metabolic and reproductive needs, this would not only enable better control of male growth rates, but could also potentially improve or maintain fertility.

It is, therefore, recommended that broiler breeder males be given a feed with a dietary crude protein content of 126 g CP/kg feed, during the peak production period. This would

possibly temper their growth rates, with the added advantage of improving or maintaining their reproductive potential. Since the recommended levels of dietary CP inclusion in female breeder diets is higher than this, it is recommended that breeder males should not be permitted to have access to the female feed, as it may have adverse effects on male fertility and semen production.

CHAPTER 5

THE EFFECT OF DIETARY SELENIUM AND VITAMIN E ON SEMEN QUALITY AND FERTILITY OF BROILER BREEDER MALES

5.1 INTRODUCTION

Spermatozoa of avian species contain high proportions of long-chain polyunsaturated fatty acids (PUFA) (Surai *et al.*, 1998). These high concentrations of PUFA make them vulnerable to lipid peroxidation, contributing to male infertility (Surai, 2002). It has been suggested that an increased dietary supplementation of Se and vitamin E could be beneficial to breeder males in maintaining the integrity of the sperm membranes (Surai *et al.*, 2000).

Studies have been conducted to determine the effect of Se supplementation in breeder male diets on different measures of fertility. Selenium can be supplied as an inorganic salt, such as sodium selenite or as part of organic molecules, such as those synthesised by Se-enriched yeast (Sel-Plex®) (Rutz *et al.*, 2005). Organic forms of selenium compounds are more active per unit of Se than inorganic salts (Ekermans & Schneider, 1982). Males given a ration supplemented with selenium yeast (0.3 mg Se/kg from Sel-Plex®) produced greater semen volume early in production than males on a ration containing sodium selenite (0.3 mg Se/kg) (Renema, 2004).

The testes of Se-deficient roosters have been shown to contain fewer hierarchies of spermatogonia that are committed to spermatid function when compared to those of Sel-Plex® fed males (Edens, 2002). Supplementation with Se has also been shown to improve sperm motility, viability and concentration (Gallo *et al.*, 2003, 2005) and the numbers of morphologically normal spermatozoa in broiler breeder males (Edens, 2002).

Vitamin E is a specific lipid soluble antioxidant in the membrane (Rutz *et al.*, 2005). It has been suggested that an increased dietary intake of vitamin E produces beneficial changes in the antioxidant capacity and lipid profile of poultry semen, maintaining the structural integrity and fertilising capacity of spermatozoa (Surai *et al.*, 1997).

In most of the studies mentioned above, male fertility was measured by semen quality traits. While these are useful measurements, a measurement that directly measures fertilising capability of the semen would be more meaningful. Thus, the number of $OPVL_{sperm}$ embedded in eggs laid by commercial layer hens, that had been artificially inseminated, were assessed, in order to determine if dietary supplementation with Sel-Plex® and/ or vitamin E would be beneficial to broiler breeder male fertility.

5.2 MATERIALS AND METHODS

Thirty two Ross 788 broiler breeder males, that had previously been used in the experiment described in Chapter 4 were allocated to this trial. They were 41 weeks of age at the beginning of the trial. The birds remained in the same raised individual cages on Ukulinga Research Farm where they had been housed during the previous experiment. The males were provided with *ad libitum* access to water throughout the experimental period.

The two basal diets that were used in the experiment described in Chapter 4 were blended together (in a ratio of 82 summit : 18 dilution) in order to obtain a feed that was used as the control diet. This blend was selected for the control diet of this trial since the males performed better on this feed during the crude protein trial (Chapter 4). After blending the two basal diets, a sample of the control diet was analysed for its nutrient contents (Table 5.1) according to the methods described in Appendix D.

Inorganic Vitamin E (at 50 percent strength) and/ or an organic form of selenium (Sel-Plex®: 1000 mg Se/kg) were added to the control diet and blended in order to obtain four treatment feeds (Table 5.2).

Since there were not enough males from the control protein group in Chapter 4 to use for this experiment, all males of each CP treatment group were randomly allocated to each of the four dietary treatments. However, since there were only 32 males (i.e. eight males per treatment) it was not possible to have an equal representation of males from each of the CP groups. The treatment groups consisted of the following number of males from each CP treatment group:-

Treatment 1: three low CP, two control CP and three high CP;

Treatment 2: two low CP, three control CP and three high CP;

Treatment 3: two low CP, three control CP and three high CP;

Treatment 4: three low CP, two control CP and three high CP.

Table 5.1 *The calculated and analysed nutrient contents (units specified) of the control diet*

Nutrient	Unit	Control Diet	
		Calculated	Actual
AMEn_adult	MJ/kg	11.09	11.08
EE	MJ/kg	9.80	
Crude protein	%	12.03	12.25
Dry matter	%	88.68	89.10
Lysine	%	0.46	0.44
Methionine	%	0.20	
Cysteine	%	0.20	
Methionine + cystine	%	0.40	0.26
Threonine	%	0.37	0.42
Tryptophan	%	0.11	
Arginine	%	0.67	0.70
Isoleucine	%	0.41	0.44
Leucine	%	1.01	1.01
Histidine	%	0.31	0.30
Phenylalanine	%	0.47	0.52
Tyrosine	%	0.36	0.31
Phenylalanine + tyrosine	%	0.83	0.83
Valine	%	0.52	0.56
Ash	%	4.09	
Crude fibre	%	5.70	
Crude fat	%	3.43	
Calcium	%	0.80	0.82
Available phosphorous	%	0.35	0.51
Sodium	%	0.16	
Chloride	%	0.16	
Potassium	%	0.70	
Linoleic acid	%	1.79	
Choline	mg/kg	1000.00	
Selenium	ng/g		190.09

Table 5.2 *Dietary treatments and their description*

Treatment	Description
1	Control feed (without additives)
2	Control + Vitamin E (0.2 g/kg)
3	Control + Sel-Plex® (0.03 g/kg)
4	Control + Vitamin E (0.2 g/kg) + Sel-Plex® (0.03 g/kg)

It was assumed that any previous effects of crude protein would be negated in this trial due to each treatment being fed to males from a range of previous CP treatments. Feed allocation was calculated, based on the mean body weight of all the birds, so as to adhere to the breeders' recommended growth curve for male parent stock (Arbor-Acres-Plus-Aviagen, 2005). This was done in order to avoid confounding of BW and dietary treatment effects. The feed was weighed out and the males were fed in the mornings. The birds were fed the experimental diets from 41 to 59 weeks of age.

The males were weighed individually at 42, 44, 47, 49, 51, 56, and 59 weeks of age. An electronic scale was used and the males were placed head-first inside a cone during weighing in order to restrain them and to avoid injuries.

After the males had been on experimental diets for a period of a week, semen was used for artificial insemination of layer hens. This was done in order to assess the ability of spermatozoa to survive in the hen (Wishart, 1987, Brillard & Bakst, 1990), and to possibly predict the fertilising ability of semen samples (Brillard & Antoine, 1990, Wishart, 1997).

Artificial insemination was performed in the late afternoon, when most of the laying hens had already laid their egg for the day (Donoghue *et al.*, 1995, Brillard, 2003). The feathers around the cloaca of each male were clipped in order to minimise contamination of the semen during collection. The insemination was performed once every two weeks until the males were 53 weeks of age. The laying hens were inseminated with 0.4 ml of diluted semen (i.e. 0.2 ml semen and 0.2 ml diluent) with an Eppendorf pipette. The semen was released slowly into the hen so as to stop semen running out of the vagina. A new pipette tip was used for the semen from each male in order to prevent any contamination of the semen samples. The decision to inseminate with a fixed volume of semen rather than a known concentration was explained in Chapter 4.

Semen was collected by the abdominal massage method (Burrows & Quinn, 1937) into small plastic flip top vials. Care was taken to avoid collecting the transparent watery fluid that is characteristically produced in the fowl (Lake & Stewart, 1978) at ejaculation in response to massage. If it is collected in abundance, it serves to dilute semen reducing the number of spermatozoa contained in a unit volume.

On each collection day, semen was collected from all of the males. This was done in order to decrease the number of days during which assistance would be required as two people were needed for both the semen collection and artificial insemination procedures. For the purpose of artificial insemination, semen was collected from four males of each treatment. Semen samples were diluted with Tyrodes immediately after being collected, before semen was collected from the next male.

Semen samples from the remaining males were used to assess semen quality. Once the semen required for the morphology and concentration assessments had been removed from the sample, the remainder of the semen was diluted with Tyrodes in order to preserve sperm motility and it was placed aside before proceeding to the next male. Once semen had been collected from all of the males, the hens were inseminated.

The day on which insemination was performed was referred to as Day 0 (D0). Eggs were collected on D2, D3, D4, D6, D7, D8 and D14 from all the inseminated hens and stored in a cold room for no more than two weeks before being assessed.

The eggs were brought to room temperature, cracked open, and the yolks were separated from the albumen. Excess albumen was removed by rolling the yolk on a paper towel. A square of approximately 1 x 1 cm of the perivitelline layer situated around the germinal disk area was then cut and removed. This piece of membrane was rinsed in PBS to remove adherent yolk, before being stretched out on a glass microscope slide. It was then stained with a 1 μg/ml solution of DAPI in PBS and covered with a cover slip (Wishart, 1987). The slides were placed in a light tight container immediately after they were stained and were examined by fluorescence microscopy within three hours.

The sperm nuclei embedded in the outer perivitelline layer in 20 randomly-chosen fields of view (each 177687.19 μm^2) were counted and totalled. These numbers were then

calculated as sperm per mm^2 of membrane. The fertility of the eggs were predicted according to two methods: one which requires more than three $OPVL_{sperm}$ per mm^2 for an egg to be considered fertile (Wishart, 1997) and another which requires 0.43 $OPVL_{sperm}$ per mm^2 for an egg to be considered fertile (Brillard & Antoine, 1990).

Besides the samples collected on the insemination days, semen was also collected at 55 and 57 weeks of age for the purpose of assessing semen quality. Semen from each male represented a replicate of its respective treatment, however not every male yielded semen on all of the collection days. On every alternate week, the males were massaged and their response was recorded, but no semen was collected. This was done in order to maintain semen production (Riaz *et al.*, 2004).

- **Morphology**

Immediately after semen collection, a drop of undiluted semen was placed within 100 µl of three percent glutaraldehyde and mixed lightly. This is done in order to fix and preserve the spermatozoa in their state at ejaculation. These samples were then kept to be examined at a later date. A drop of the sample was placed onto a microscope slide and gently covered with a cover slip before being examined with a light microscope at 40 times magnification. Three hundred spermatozoa on each slide were counted at random and defects were noted and then calculated as a percentage of the total observed. The following defects could be noted: sperm bent at head-midpiece or midpiece-tail junctions, knots or swelling at head-midpiece junctions or in the head region, and coiled heads (Lake & Stewart, 1978, Alkan *et al.*, 2002).

- **Concentration**

Prior to semen collection, solutions containing Eosin and Nigrosin were prepared for each semen sample. A known volume of undiluted semen (either 50 or 100 µl, depending on the volume of semen yielded by the bird) was placed within the Eosin and Nigrosin solution and mixed well by rotating the bottles gently. A small amount of the mixture was then used to fill the two sides of a haemocytometer. Spermatozoa were counted in 10 random blocks on either side of the haemocytometer at 40 times magnification using a light microscope.

This data, together with the corresponding mass motility scores, were then used in a formula (Appendix C) to calculate the number of live sperm per ml of semen.

- **Motility**

After removing the semen needed for the concentration and morphology assessments, the remainder of each semen sample was immediately diluted with Tyrodes solution and placed aside before proceeding to the next male. On the days on which artificial insemination was done, the motility assessment was only done after all of the hens had been inseminated. On the remainder of the semen collection days, the motility assessment was done immediately after semen had been collected from all of the males. A drop of the diluted semen sample was placed on a pre-warmed slide and gently covered with a cover slip. A light microscope at 40 times magnification was used to examine the sample. Three random areas on the slide were examined in order to score the percentage progressive motile normal sperm, the percentage abnormal motile sperm and the percentage dead sperm. Care was taken to minimise the time between collecting and analysing the semen motility.

At the end of the experiment, the response of the males to abdominal massage was assessed and body weights were recorded. The birds were slaughtered on the following day. After slaughter, the abdominal cavity was opened, and the testes were removed. The presence or absence of semen within the vas deferens was noted. The epididymal tissue was carefully cut off and each testis was weighed. The length and the width of both the left and right testes were measured.

Statistical Analysis

The frequency of eggs considered fertile per male dietary treatment according to the predictions of Wishart (1997) (W) and of Brillard & Antoine (1990) (BA) were subjected to a Chi-Square test of independence using *Minitab* (Minitab-Inc., 2007). This was done in order to test the null hypothesis that fertility and dietary treatments were not associated.

Unless stated otherwise, *GenStat 11th Edition* (Lawes-Agricultural-Trust, 2008) was used for all further data analyses as well as any graphical output.

The numbers of $OPVL_{sperm}$ observed in all of the eggs collected from laying hens on D2, D3, D4, D6, D7, D8 and D14 post-insemination were subjected to a general ANOVA with an unbalanced treatment structure. This type of ANOVA was used as an unequal number of eggs represented each dietary treatment group. Significant effects of age ($P<0.05$) and day post-insemination ($P<0.001$) on the number of $OPVL_{sperm}$ were observed, and thus these were treated as "nuisance terms" in the ANOVA model, in order to focus on the treatment effects. The $OPVL_{sperm}$ means were compared using the appropriate LSD values at 5 percent level of significance. Exponential curves were fitted with standard curve regression to observe the relationship between $OPVL_{sperm}$ and day post-insemination.

The percentage fertility of eggs obtained from laying hens was predicted from the median $OPVL_{sperm}$ per mm^2 values of each group at different days post-insemination (Staines *et al.*, 1998). The calculated percentage fertility data were subjected to a general ANOVA and differences in overall dietary treatment means were compared by the LSD value at 5 percent level of significance.

The semen morphology, concentration, motility and number of live sperm per ml of semen data were subjected to a general ANOVA with an unbalanced treatment structure. The treatment structure was unbalanced due to the different number of birds from each treatment that yielded semen on the collection days. All means were compared using the appropriate LSD values at 5 percent level of significance.

The counts of positive and negative responses of the males on each dietary treatment, to the abdominal massage method, were recorded during the course of this trial. The total counts were subjected to a Chi-Square test of independence using *Minitab* (Minitab-Inc., 2007). This was done in order to test the null hypothesis that semen yield and dietary supplementation treatments were not associated.

The testes weights were subjected to a general ANOVA with an unbalanced treatment structure as there was an unequal number of males in each treatment group (one male went missing some time between the age of 57 and 59 weeks). The dietary treatment means were compared with the appropriate LSD value at 5 percent level of significance.

The left testis weight as a percentage of total testis weight was calculated to investigate whether there was any difference between the weights of the left and right testes. A mean of 0.482 was found for left testis weight as a percentage of total testis weight. This indicated that the left and right testes did have different weights. Left and right testis weights were then compared using a paired *t*-test in order to determine whether this difference was significant. The relationship between left and right testis weights was then further investigated using simple linear regression analysis. Simple linear regression analysis was also used to determine the relationships between length or width with testis weight. Multiple linear regression was used to examine the relationship between length and width with testis weight.

The presence or absence of semen in the vas deferens of the males on each treatment were recorded at the end of the trial. The total counts were subjected to a Chi-Square test of independence using *Minitab* (Minitab-Inc., 2007). This was done in order to test the null hypothesis that the presence of semen in the vas deferens of the males, and dietary supplementation treatments, were not associated.

5.3 RESULTS AND DISCUSSION

The Chi-Square values obtained from the counts of fertile and non-fertile eggs were as follows: 3.994 (P = 0.262) and 2.464 (P = 0.482), for W and BA respectively. The null hypothesis was thus accepted in both cases and no further tests were done between the dietary treatments.

No significant differences were observed with regards to the number of $OPVL_{sperm}$ per mm^2 between dietary treatment groups. There was a significant (P<0.001) effect of day post-insemination on the number of $OPVL_{sperm}$ within oviposited eggs across all dietary

treatments (Table 5.3). Eggs on D2 contained a significantly higher amount of $OPVL_{sperm}$ than any of the other days post-insemination. Numbers of $OPVL_{sperm}$ in eggs collected on D3 and D4 were significantly higher than in eggs of D6, D7 and D8. Eggs collected on D14 contained significantly lower numbers of trapped sperm than on any of the other days post-insemination.

Table 5.3 *Mean values (± s.e.m.) of $OPVL_{sperm}$ per mm^2 of eggs obtained from laying hens on D2, D3, D4, D6, D7, D8 and D14 post-insemination*

Day post-AI	$OPVL_{sperm}$ per mm^2
2	$6.1^{a} \pm 0.3$
3	$3.9^{b} \pm 0.3$
4	$2.9^{c} \pm 0.3$
6	$1.8^{d} \pm 0.3$
7	$1.7^{d} \pm 0.3$
8	$1.4^{d} \pm 0.3$
14	0.5 ± 0.3

^{a,b,c,d} Means in a column followed by different superscripts are significantly different at 5 percent level of significance

Separate exponential curves ($P<0.001$; estimated S.E. = 3.49, 3.31, 3.14 and 3.27, for treatments 1, 2, 3, and 4 respectively) were fitted to the $OPVL_{sperm}$ per mm^2 data of each dietary treatment (Figure 5.1). The y-axis scale of Figure 5.1 was narrowed in order to make the individual points more visible (Figure 5.1b). It is evident that there were only slight differences between the dietary treatments with regards to the number of $OPVL_{sperm}$ throughout the 14 day collection period. The value of y = 3 was substituted into each of the exponential curve equations in order to determine the duration of fertility (Wishart, 1997). It would appear that the duration of fertility was 3.54, 4.12, 4.45 and 3.74 days in eggs inseminated with semen from males on treatment 1, 2, 3 and 4, respectively. Therefore, dietary supplementation with either vitamin E or Sel-Plex®, as well as a combination of the two supplements, appears to have a beneficial effect on the duration of fertility. It has been suggested that dietary supplementation with Se and vitamin E exerts a positive effect on fertility in aging hens, by increasing their capabilities for sperm retention in the SST (Breque *et al.*, 2003, 2006). Renema (2004) observed a higher incidence of sperm holes in the PVL of oviposited eggs when both the male and female diets were supplemented with 0.3 mg Se/kg form Sel-Plex®. The slightly higher $OPVL_{sperm}$ numbers observed in the

groups that were supplemented with either vitamin E or Se, and the longer duration of fertility in all of the supplemented groups, than in the control group, may therefore suggest that there is some benefit in supplementing the diets.

It is evident from Figure 5.1b that there is a single observation on D14 that is above all of the other observations. Upon further investigation, it was found that the semen sample used to inseminate the laying hen that laid that particular egg (which contained 2.81 $OPVL_{sperm}$ per mm^2), was also used to inseminate two other laying hens. Interestingly, though, one of the other two eggs collected on D14 post-insemination had no $OPVL_{sperm}$, whereas the other contained 0.56 $OPVL_{sperm}$ per mm^2. This emphasises the effect that the hen could have on the duration of fertility, depending on her ability to store semen within the SST, as well as the rate at which sperm are released from the SST. It is, therefore, important that any comparisons made between treatments, with regards to fertility as determined by the number of $OPVL_{sperm}$, should be done with as many replicates as possible.

No significant dietary treatment effects were observed on sperm morphology (Table 5.4). This may be because vitamin E and Se were naturally occurring in adequate quantities in the raw materials used in the diet formulation to have a positive effect, even on males on the control diet. There were also no significant dietary effects on the sperm motility, concentration or number of live sperm per mm^2 (Table 5.5). There is controversy in the literature, with some experiments resulting in a positive effect of vitamin E (200 mg/kg diet) and Se (2.5 mg/kg LW of Se as 1 percent NaSe) supplementation on sperm quality traits (Gallo *et al.*, 2003, 2005, Singh & Nagra, 2008), while Selvan (2007) reported no significant differences in semen quality traits when breeder males were supplemented with different amounts of vitamin E (10 versus 40 mg/kg diet). This may also arise due to experiments not being published when no significant differences between treatments are found, especially if such trials are commissioned by feed additive companies.

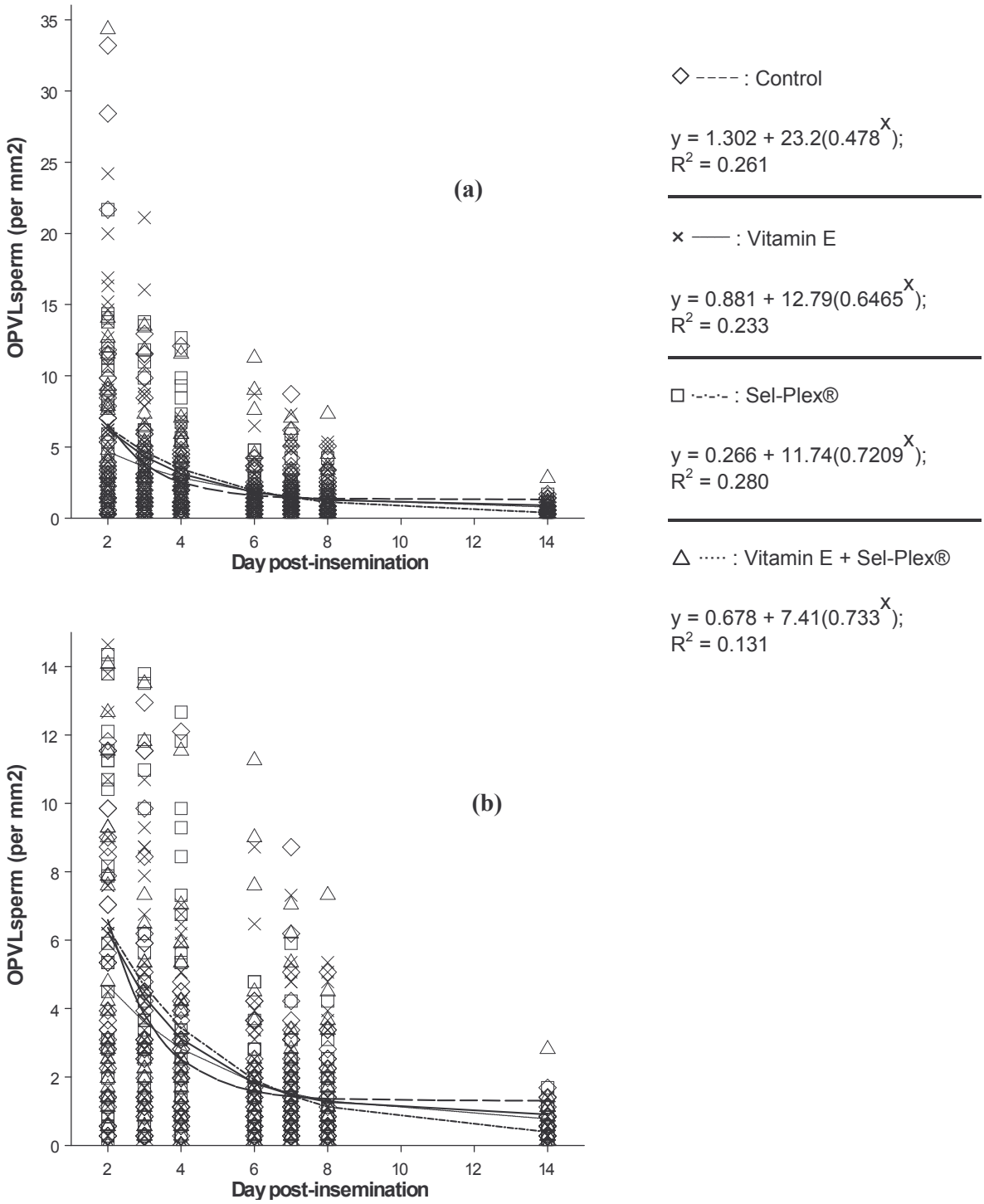

Figure 5.1 *Number of $OPVL_{sperm}$ per mm^2 of eggs obtained from laying hens on D2, D3, D4, D6, D7, D8 and D14 post-insemination.*

Table 5.4 *Mean values of morphology (± s.e.m) of semen obtained from Ross broiler breeder males fed different dietary treatments from 41 to 59 weeks of age*

	Morphology (%)						
Dietary treatment	Normal	Bent	Coiled heads	Knots in head	Knots at head-midpiece junction	Swelling in head	Swelling at head-midpiece junction
Control	94.9 ± 1.2	3.1 ± 0.7	0.3 ± 0.4	0.5 ± 0.2	0.4 ± 0.2	0.5 ± 0.2	0.5 ± 0.6
Vitamin E	94.7 ± 1.2	3.8 ± 0.7	0.7 ± 0.4	0.2 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.1 ± 0.6
Sel-Plex®	92.2 ± 1.1	4.4 ± 0.6	0.4 ± 0.3	0.6 ± 0.2	0.5 ± 0.2	0.3 ± 0.2	1.6 ± 0.5
Vitamin E + Sel-Plex®	92.3 ± 1.5	4.7 ± 0.8	0.6 ± 0.5	0.5 ± 0.2	0.5 ± 0.2	0.6 ± 0.2	0.9 ± 0.7

Table 5.5 *Mean values (± s.e.m.) of motility, semen concentration and numbers of live sperm per ml of semen obtained from Ross broiler breeder males fed different dietary treatments from 41 to 59 weeks of age*

	Motility (%)				
Dietary treatment	Progressive normal motile	Abnormal motile	Non-motile (dead)	Concentration sperm/ ml ($x10^6$)	Number of live sperm/ ml ($x10^6$)
Control	69.0 ± 4.8	12.9 ± 1.7	18.1 ± 4.3	151.2 ± 16.1	133.3 ± 15.7
Vitamin E	64.0 ± 4.5	12.3 ± 1.6	23.7 ± 4.0	113.6 ± 15.8	95.5 ± 15.1
Selplex	65.6 ± 4.2	14.6 ± 1.5	19.8 ± 3.7	130.1 ± 14.5	106.3 ± 13.9
Vitamin E + Selplex	59.2 ± 4.8	13.7 ± 1.7	27.1 ± 4.3	133.7 ± 16.8	112.0 ± 16.0

The Chi-Square value obtained from the total counts of positive and negative responses of the males in each dietary treatment group to semen collection, during the entire duration of the trial, was as follows: 0.510 (P = 0.917). The null hypothesis was thus accepted and no further tests were done between the treatment groups with regards to the frequency of semen yielded. The percentages of males in each treatment group that yielded semen in response to abdominal massage at various ages during the trial was calculated (Table 5.6). It is evident that the number of males that responded positively to semen collection was generally lower in all of the treatment groups at 58 and 59 weeks of age. It is possible that the testes of some of the males may have started to regress, as the birds were at the end of their production period.

Table 5.6 *Mean percentage (± s.e.m) of males that produced semen in response to abdominal massage at various ages during the trial period*

Age (weeks)	Males producing semen (%)			
	Control	Vitamin E	Sel-Plex®	Vitamin E + Sel-Plex®
42	100	87.5 ± 12.5	100	87.5 ± 12.5
43	100	87.5 ± 12.5	87.5 ± 12.5	100
46	100	100	100	100
47	87.5 ± 12.5	100	62.5 ± 18.3	75.0 ± 16.4
48	100	100	100	87.5 ± 12.5
50	100	100	87.5 ± 12.5	75.0 ± 16.4
52	100	75.0 ± 16.4	75.0 ± 16.4	100
54	75.0 ± 16.4	87.5 ± 12.5	100	100
56	75.0 ± 16.4	100	75.0 ± 16.4	75.0 ± 16.4
58	50.0 ± 18.9	62.5 ± 18.3	87.5 ± 12.5	50.0 ± 18.9
59	50.0 ± 18.9	25.0 ± 16.4	62.5 ± 18.3	57.1 ± 20.2
Mean over entire period	85.2 ± 3.9	84.1 ± 3.9	85.2 ± 3.9	82.8 ± 3.9

In order to investigate the possibility of regressed testes in the males, the birds were euthanased at the age of 59 weeks. The testes of some of the males had clearly regressed as they were very small and had a poor coverage of blood vessels (Figure 5.2a), when compared to testes that had not regressed (Figure 5.2b). Some of the males also clearly had no semen in the vas deferens as the size of these ducts were reduced and they were pale in colour (Figure 5.3a), when compared with the vas deferens of males that were thick and milky in colour (Figure 5.3b) however this was unrelated to treatment.

There was no significant treatment effect on the left, right, total or average testis weights of males on the different treatments (Table 5.7). However, the males in the control group, had higher average testes weights than the dietary supplemented groups, which, if not a dietary response, could have had an influence on the slightly improved semen quality traits seen from males on the control diet. The vitamin E treatment appears to have lower testes weights than the other groups. It has been found that testes weights, density of sperm (per µl), and sperm morphology were negatively influenced by very high doses of vitamin E (Danikowski *et al.*, 2002). The lower testes weights of the birds in the vitamin E supplemented group may possibly suggest that the birds may have been given too much vitamin E. This is possible, since the raw feed ingredients of the control diet would also have contained some vitamin E. However, the level of vitamin E in the control diet was not determined and thus it would not be correct to make any definite conclusions with regards to the possible excess level in the supplemented diet.

Table 5.7 *Mean testes weights (g) (± s.e.m.) of males at 59 weeks of age*

	Mean testes weights (g)			
Dietary treatment	Left	Right	Total	Mean
Control	10.1 ± 1.6	10.8 ± 1.5	20.9 ± 3.0	10.5 ± 1.5
Vitamin E	7.7 ± 1.6	8.3 ± 1.5	15.9 ± 3.0	8.0 ± 1.5
Sel-Plex®	10.0 ± 1.6	9.9 ± 1.5	19.9 ± 3.0	10.0 ± 1.5
Vitamin E + Sel-Plex®	8.8 ± 1.8	9.9 ± 1.6	18.6 ± 3.2	9.0 ± 1.6

The right testis weights were greater than that of the left testis, although the difference was not significant (P = 0.062). The null hypothesis that was tested by the paired *t*-test was that mean left testis weight less mean right testis weight is equal to zero. The mean value of this test was found to be -0.5761, which indicates that right testis weight was in fact greater than left testis weight. This finding is in disagreement with Bennett (1947), who in a study of Single Comb White Leghorns, found the mean left testis weight to be greater than that of the right testis weight in ten out of twelve groups of males studied. However, in another study of testicular asymmetry in broiler breeders, the left, as a proportion of total testis weight was found to be approximately normally distributed about a mean of 0.515 and the left testis weight was also greater than the right testis weight in 67 percent of the males (Hocking, 1992). According to Etches (1996), the left testis is usually 0.5 to 3 g larger than the right. However, in a study of Red Jungle fowl (*Gallus gallus*), no tendency was found

for the left testis to be larger than the right (Kimball *et al.*, 1997), which is in agreement with the observed results of the current experiment.

After regression of left (*Y*) on right (*X*) testis weight the following equation was obtained: $Y = -0.472 (\pm 0.0753) + 0.9893 (\pm 0.792) X$. This accounted for a large proportion of the variance ($R^2 = 0.851$), showing that there is a consistent relationship between left and right testis weights. This is consistent with Hocking (1992), who found a high correlation coefficient ($r = 0.98$) for the regression of $\log_e$ left on $\log_e$ right testis weight.

The remaining regression equations involving testes weights and dimensions with the corresponding percentage variances accounted for are shown in Table 5.8. It appears that the left testis length is a better indicator of testis weight than the width. The reverse is true in the case of the right testis, where the width is a better indicator of testis weight than the length. Also, when looking at both testis length and width, the percentage variance accounted for by the equations improve. The significance of this fact depends on the degree of accuracy required when predicting testis weight from testis dimensions as well as the time available to do the estimate. It would be recommended, though, that both the length and the width of the right testis be measured in order to more accurately predict testis weight. This may be of practical use when testis weight needs to be estimated without sacrificing the bird. Ultrasound may be used to measure either just the length or width, or both length and width of the testis (Richardson *et al.*, 2002) and the regression equations can then be used to calculate the weight.

The Chi-Square value obtained from the total counts of males in each group that had semen in their vas deferens at 59 weeks of age, was as follows: 1.365 ($P = 0.714$). The null hypothesis was thus accepted and no further tests were done between the dietary treatments, with regards to the effect of male treatment on the presence of semen in the vas deferens. The proportions of males that had semen in their vas deferens were 0.625 (±0.1843), 0.375 (± 0.1843), 0.625 (± 0.1843), and 0.5741 (± 0.1771), of the birds in treatment 1, 2, 3, and 4, respectively. It is thus evident that most of the males that were on the vitamin E supplemented diet, were no longer producing semen. This is due to a higher

incidence of regressing testes, as seen by lower testes weights. In the current experiment, all the males with an average testis weight below 8.1 g were no longer producing semen.

Table 5.8 *Equations obtained using regression analysis of testes weight, length and width*

Regression	Equation	P-value and estimated S.E.	Variance accounted for (%)
L*_weight (*Y*) L_length (*X*)	$Y = -10.01\ (\pm 1.97) + 4.771\ (\pm 0.481)\ X$	<0.001 2.12	76.4
L_weight (*Y*) L_width (*X*)	$Y = -0.21\ (\pm 2.88) + 4.24\ (\pm 1.27)\ X$	<0.05 3.78	25.3
R**_weight (*Y*) R_length (*X*)	$Y = -12.07\ (\pm 2.18) + 5.175\ (\pm 0.511)\ X$	<0.001 1.95	77.2
R_weight (*Y*) R_width (*X*)	$Y = -9.70\ (\pm 1.49) + 8.968\ (\pm 0.677)\ X$	<0.001 1.57	85.3
L_weight (*Y*) L_length (*X*) L_width (*Z*)	$Y = -10.91\ (\pm 2.07) + 4.433\ (\pm 0.546)\ X + 1.021\ (\pm 0.810)\ Z$	<0.001 2.10	76.9
R_weight (*Y*) R_length (*X*) R_width (*Z*)	$Y = -13.27\ (\pm 1.27) + 2.446\ (\pm 0.462)\ X + 5.858\ (\pm 0.763)\ Z$	<0.001 1.13	92.4

* L represents left testis

** R represents right testis

As mentioned before, there is a natural decline in fertility of broiler breeders after the peak production period. Poor body weight management or a sudden loss of body weight could, however, accelerate the natural process of testes regression (Powley, 2008). The males in the current study were weighed at 51 weeks of age, and then again at 56 weeks of age, and no adjustments to the feed allocation were necessary within that time. At 56 weeks of age, a mean loss in body weight of 343 g was observed, after which the feed allocation was increased. The general decrease in the number of males that yielded semen in response to abdominal massage at 58 weeks of age, could therefore, have been an indication of testes regressing in some of the males, possibly due to the inadequate feed allocation in the previous weeks. This emphasises the importance of proper body weight monitoring of broiler breeder males, throughout the entire production stage, and perhaps even before that.

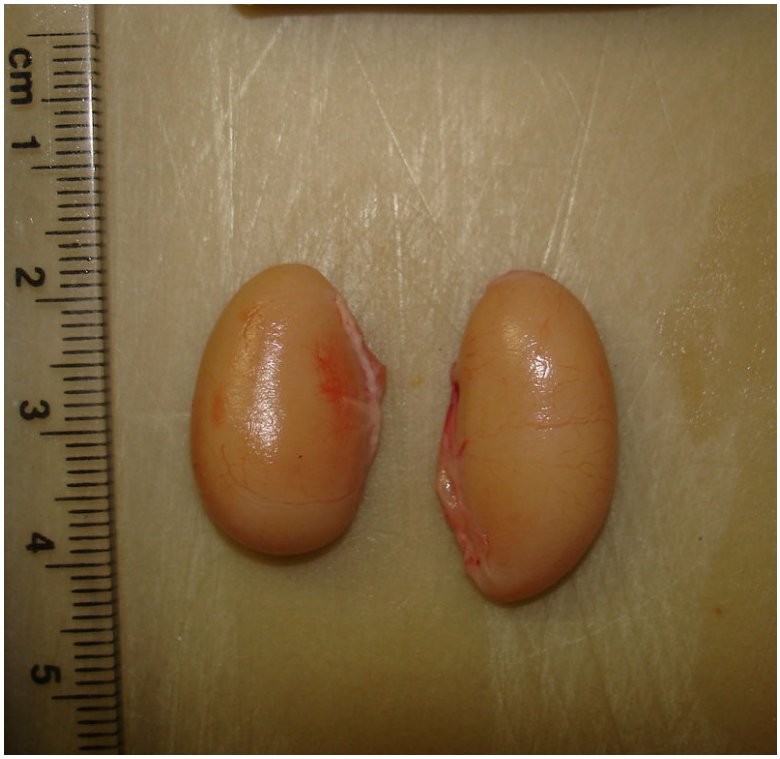

Figure 5.2a *Regressing testes of a breeder male at 59 weeks of age.*

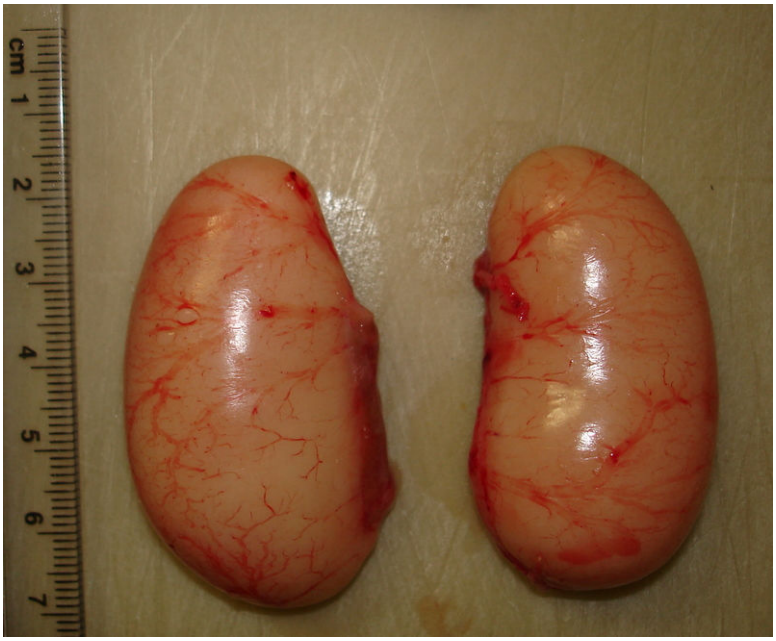

Figure 5.2b *Testes of breeder male at 59 weeks of age that were still functioning.*

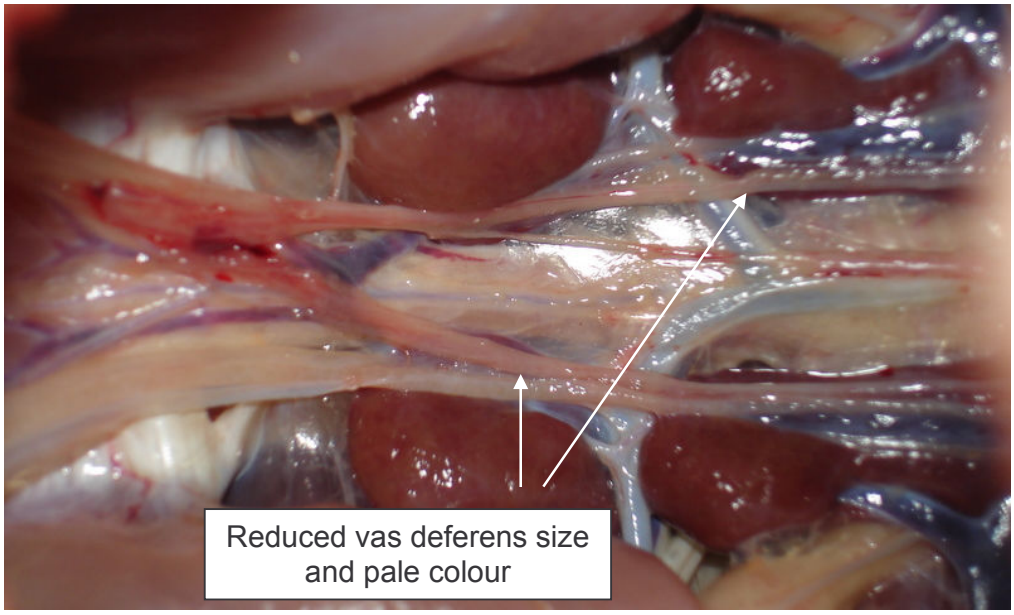

Figure 5.3a *Vas deferens of a male that was no longer producing semen at 59 weeks of age.*

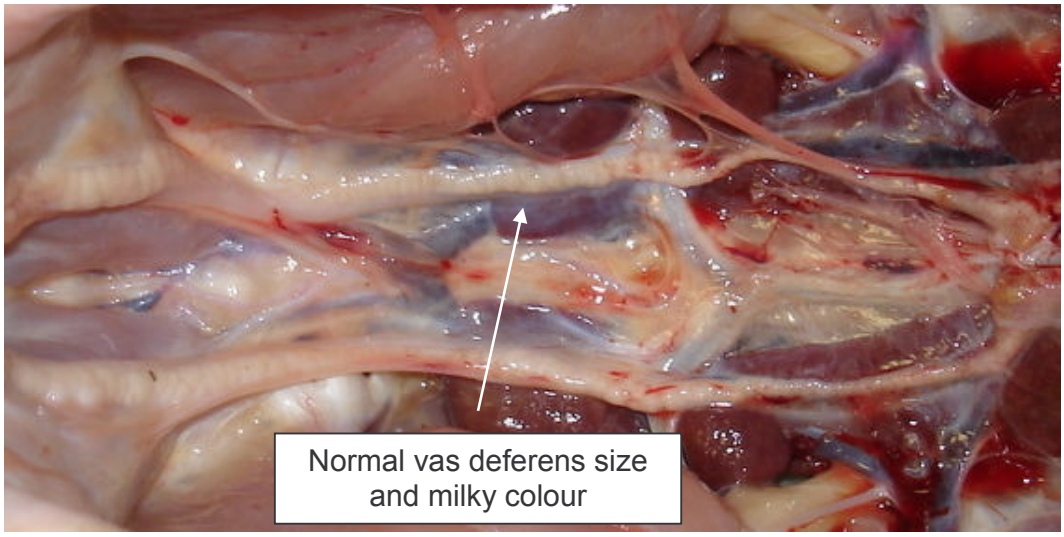

Figure 5.3b *Vas deferens of a male that was still producing semen at 59 weeks of age.*

5.4 CONCLUSIONS

No significant effects of supplementing breeder male diets with additional vitamin E or Sel-Plex® were observed. The slightly higher duration of fertility in the eggs obtained from hens inseminated with semen of the Se and vitamin E supplemented males, would suggest that there may have been a slight improvement in the fertilising ability of the spermatozoa or an increased ability to survive within the reproductive tract of the hens. The improvement, however, was not observed in semen quality traits. However, the fertilising potential of the sperm is thought to be more accurately judged through the sperm in the OVPL rather individual measures of semen quality (Wishart & Staines, 1999, Wishart *et al.*, 2001). It is possible that the levels of these nutrients present in the control feed may have already been sufficient to cause an improvement in sperm fertilising ability so that no differences were observed between treatments. The males were 42 weeks old at the beginning of this experiment. It is suggested that future studies could perhaps investigate the effects of earlier supplementation with additional vitamin E or Se on male semen quality and fertility. The importance of proper body weight management has also been emphasised.

CHAPTER 6

GENERAL CONCLUSIONS

The decline in fertility of broiler breeders is a concern to the industry, and investigations into the role of management and the environment in improving fertility are of importance. The aim of this thesis was to assess nutritional effects in the form of crude protein, vitamin E and Se, in the form of Sel-Plex®, on fertility of the male broiler breeder in order to make recommendations to maximise male breeder fertility.

Current breeder recommendations for CP levels in feed are 120 to 140 g/kg during the production stage. Males require less protein than females who need to support egg production, and with the advancement in separate-sex feeding it has become possible to feed different rations to males and females, however, it is possible that this is not being implemented in all cases, in which case males are receiving females feed with typical CP levels of 145 to 155 g/kg. The results of this study not only clearly demonstrates the benefits of giving males lower CP feeds (126.1 g/kg CP), but they also suggest that high CP diets (i.e. 150 g/kg CP) may negatively influence male fertility. It is recommended that males should not be given a feed that has been formulated to meet the requirements of the breeder hens. However, further research may need to be done on levels of CP between 126 and 150 g/kg CP, as there may well be a better response on a level between those two levels of inclusion. However the relationship does appear to be quadratic with negative effects of low CP being observed.

Despite reported benefits of supplementation with additional organic Se or vitamin E (Surai, 2000, Edens, 2002, Breque *et al.*, 2003, Gallo *et al.*, 2003, Renema, 2004, Gallo *et al.*, 2005, Breque *et al.*, 2006, Singh & Nagra, 2008), no benefit to male fertility was observed at the levels of inclusion used in the current experiment. Therefore, further research needs to be done in this field, possibly with a range of inclusions of each vitamin E and Se, as this was not done in the present study. It may also be worth investigating the effect of these supplements, when given to the males from a younger age. It is also worth considering the possibility that other studies may also have shown no significant differences, with regards to feed supplementation with either vitamin E or Se. The findings of these studies may possibly not have been published, especially when the experiments

were research commissioned and possibly paid for by particular feed additive companies. The possibility also exists that raw materials used in animal feeds could vary in levels of vitamin E and Se, and that perhaps their resultant levels of inclusion in the control feed of the current experiment were already in a range that would improve reproduction in the males.

Artificial insemination and semen quality analysis in poultry had not been performed much at the University of KwaZulu-Natal in recent years before this experiment. It was therefore considered necessary to practice and perfect various experimental techniques before any trial work was performed, and thus, a pilot study was undertaken to ensure fertile eggs after AI, as well as to obtain practice in feed formulation and mixing of feed. A number of factors were highlighted during the course of this preliminary trial, which were taken into consideration in all the experimental work done thereafter.

Appendix A

Composition of the Tyrodes solution.

Component	Quantity (g)
$CaCl_2$	0.2 *
$MgCl_2 \cdot 6H_2O$	0.1
KCl	0.2
$NaHCO_3$	1.0
NaCl	8.0
NaH_2PO_4	0.04 anhydrous / 0.052 hydrous
Glucose	1.0
Distilled water	up to 1000 ml

* add last with stirring to prevent precipitation

The solution must be filtered into a sterile container, stored in a refrigerator and discarded after two weeks, as it is an ideal medium for supporting growth of bacteria and moulds.

Appendix B

Composition of the Eosin and Nigrosin solution.

- 8 ml distilled water
- two drops Eosin *
- five drops Nigrosin **

* Eosin – 5 g per 100 ml distilled water

** Nigrosin – 10 g per 100 ml distilled water

Appendix C

Formula used to calculate the number of live sperms per ml of sample.

$$B \times \frac{1000^*}{0.004} \times \frac{1}{A} \times \frac{C}{1} \times \frac{D}{100}$$

$$= \frac{B \times C \times D}{A} \times 2\,500$$

A- Total number of blocks counted (e.g. 20)

B- Total number of sperms counted (e.g. 64 in 20 blocks)

C- Dilution factor (e.g. 0.1 ml in 8.0 ml gives a factor of 80)

D- Motility of the sperms (e.g. 65/5/30 gives 65+5=70)

(Therefore count the percentage progressive motile normal sperm and the percentage motile abnormal sperm together to get D)

$^*\frac{1000}{0.004}$ = factor to convert volume of blocks in haemocytometer to ml.

Example:

$$\frac{64 \times 80 \times 70}{20} \times 2\,500$$

= 44.8 million live sperm/ ml semen

Appendix D

Methods used to determine nutrient compositions of experimental feeds.

Nutrient	Reference of method used
AMEn_adult	(McNab & Fisher, 1984)
Crude protein	(AOAC Official Method 990.03)
Moisture	(AOAC Official Method 934.01)
Amino acids	(Moore & Stein, 1984)
Ash	(AOAC Official Method 942.05)
Crude fibre	(AOAC Official Method 978.10)
Crude fat	(AOAC Official Method 920.39)
Calcium	(AOAC Official Method 968.08)
Available phosphorous	(AOAC Official Method 965.17)
Selenium	(Koh & Benson, 1983)

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