THE EFFECTS OF DIETARY CRUDE PROTEIN ON FERTILITY OF BROILER BREEDER MALES

By

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Submitted in fulfilment of

the requirements for the Degree of

MASTER OF SCIENCE IN AGRICULTURE

in the Discipline of Animal & Poultry Science

School of Agricultural, Earth and Environmental Sciences

College of Agriculture, Engineering and Science

University of KwaZulu-Natal

Pietermaritzburg

ABSTRACT

Fertility of Broiler Breeders (BBs) has been researched over many years with inconclusive findings. Genetic selection and improvements in growth traits have had negative effects on fertility of BB. One explanation is related to overweight birds, resulting in the inability of birds to copulate successfully, with no cloacal contact. The requirements of CP for egg production in BB females have been quantified, but there is less literature on how CP affects fertility in male BBs. The objective of this experiment was to investigate the effects of feeding BB males different dietary CP intakes (10.4, 12.4, 14.2, 17.8, 19.3 and 20.1 g CP/bird/day) on fertility in flocks with natural mating and in birds that were artificially inseminated (AI). Results showed that birds consuming 10.4 and 12.4 g CP/bird/day resulted in weight loss (between 24.9 and 23.6% and 26.5 and 22.4% below target BW) over the 29 weeks of the experimental period in both natural mating and individual pens respectively. Intakes of higher protein content (17.8-20.1 g CP/bird/day) however resulted in BW gain over the experimental period. Hatch percentage across all CP intakes throughout the duration of the study was found to be high, ranging between 75- 100 and 80-95 % in AI and naturally mated birds respectively, and not affected by CP intakes. Similar results for predicted fertility using the methods published by Brillard & Antoine (1990) and Wishart (1997) were found; however Brillard & Antoine (1990) is more lenient requiring less outer perivitelline layer (OPVL) sperm/mm^2 to pronounce an egg fertile. Improved fertility predictions could have been made by using examination of inner perivitelline layer (IVPL).

There was a tendency for a superior response in predicted fertility from birds with a protein intake of 14.2 g CP/bird/day. This intake also least affected the BW of the male birds and thus 14.2 g CP/bird/day can be recommended for optimal BB male performance.

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 February 2010 to December 2010, under the supervision of Dr Nicola C. Tyler and Dr M Ciacciariello.

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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Signed: …….

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following people and institutions for their contributions to this thesis:

Dr Nicola Tyler for her patience, assistance, enthusiasm and commitment throughout this experimental period.

Dr Mariana Ciacciariello for her guidance, encouragement, endless input and suggestions.

The National Research Foundation for a Thuthuka grantholder-linked bursary.

The Protein Research Fund for a Post Graduate Scholarship in Research on Protein.

Anne Kinsey and the staff at the Ukulinga Research Farm for their assistance and dedication for the duration of the experiment.

Jamila Patel, for the laughs and hard work and friendship throughout the trial.

My mom for her unconditional love, guidance, support and encouragement in everything I do.

The rest of my friends and family, for their continuous love, support and devotion.

Most importantly, the Lord, for giving me this opportunity and guiding me every step of the way.

TABLE OF CONTENTS

ABBREVIATIONS USED IN THE TEXT

- µl- micro litre
- AA(s)- amino acid(s)
- AI- artificial insemination
- AOAC- Association of Analytical Chemists
- ANOVA- analysis of variance
- BB(s)- broiler breeder(s)
- BW(s)- body weight(s)
- CP- crude protein
- D(0,2,3,4)- days 0, 2, 3 and 4
- DAPI- diamidinophenylindole
- E- energy
- FCE- food conversion efficiency
- G- gram
- IPVL- inner perivitelline layer
- kg- kilogram
- LED- limited every day
- LSD- least significant difference
- ME- metabolisable energy
- MJ- mega joules
- Ml- millilitres
- N- nitrogen
- NRC- National Research Council
- OPVL- outer perivitelline layer
- $OPVL_{sperm}$- sperm embedded in the outer perivitelline layer
- $OPVL_{sperm}/mm^{2}$- sperm embedded in the outer perivitelline layer per millimeter^2
- P- probability
- PBS- phosphate buffered saline
- SAD- skip- a- day
- SE- standard error
- SST(s)- sperm-storage tubule(s)

- UVJ- utero-vaginal junction

CHAPTER 1

GENERAL INTRODUCTION

The fertility of eggs is dependent on both hen and cockerel fertility, as explained by Hays (2007), where physical characteristics play a lesser, but also important role in hatchability than the genetic make-up of the bird. A natural function of a reproducing BB is to produce viable embryos which mature and result in marketable broilers.

A number of researchers have performed various experiments in order to establish the nutrient requirements of the BB female and the effects of maternal nutrition. A developing embryo and the hatched chick are solely dependent on nutrients deposited in the egg and therefore a balanced nutritional status of the breeding stock is imperative for optimal embryo development and chick viability (Wilson, 1997; Kenny & Kemp, 2005). Sufficient nutrients need to be transferred to the egg for normal embryo growth and development. This transfer of nutrients largely depends on maternal diet and metabolism (Wilson, 1997). Kenny & Kemp (2005) explain that a high protein: energy ratio reduces hatchability and chick performance within a flock. The level of restriction severity, deficiencies, excesses and imbalances of nutrients may be fatal to the developing embryo (Wilson, 1997).

In today's society, an ever-increasing population has resulted in a greater demand for commodities. This has consequently resulted in intensive genetic selection for birds that grow at a more rapid rate. The major traits selected for in broilers and BBs are BW and food conversion efficiency (FCE). Selection has not only affected metabolic traits but also reproduction, health and the overall behaviour of the birds (Anonymous, 2000). Higher meat yield, improved feed efficiency and rapid broiler growth rates have resulted in a swift modification of broiler chickens, resulting in distorted muscular distribution, excessive BW and a reduced ability to mount and therefore copulate successfully (McGary *et al.,* 2003). Observations from experiments conducted by Soller & Rappaport (1971) and Lake (1989) show that reproductive traits of broilers and layers are negatively correlated to BW and growth rates.

Although modern broilers have an improved FCE (less food required per unit of BW gain) and a decreased slaughter age, these modifications have rendered them unable to regulate feed intake (Bokkers & Koene, 2003). This over-consumption of food in the parent stock results in obesity, increased carcass fat and reduced productivity due to the lack of successful cloacal contact during mating (Nir *et al.*, 1978; Siegel & Dunnington, 1985; Emmerson, 1997). Providing unlimited access to feed in the parent stock results in consumption in excess to that required for maintenance and production; the outcome being that surplus E is converted to fat. It is therefore imperative to maintain health and reproductive fitness within the broiler parent stock. Highly structured feed restriction programmes, have been set in place to control the weight of these birds, especially that of the male.

Crude protein intakes of male and female BB rations differ due to higher requirements of females for egg production. Separate sex feeding has made it possible to feed different diets. However, in some BB operations, males are fed the same feed as the females, only with varying degrees of feed restriction. It is important to determine whether the degree of restriction of CP influences fertility of natural-mating BB males during the reproductive period. Crude protein contributes to a large portion of expense in feed formulation and therefore it is imperative to determine the optimum intake from both a fertility and cost point of view. Lopez & Leeson (1995) found that variations of CP and amino acid (AA) contents have an effect on the nitrogen (N) content of the excreta; where N increases as CP levels increase, so overfeeding protein also constitutes an environmental concern.

Limited, inconclusive, literature is available in establishing nutrient requirements of BB males; CP in particular. The effects of this on fertility have been investigated with inconsistent findings, probably due to the variation in the nature of assessment of male fertility.

The aim of this experiment was to review the literature on the effects of CP on BB fertility and to determine the optimum intake of protein using a CP range that is both below (10.4, 12.4 and 14.2 g CP/bird/day) and within (17.8, 19.3 and 20.1 g CP/bird/day) recommended values specified by breeder companies to maximise male BB fertility.

CHAPTER 2

LITERATURE REVIEW

The drawback of the high selection pressure for growth and production in BBs is the decline in fertility that results in a loss of hatching eggs. Possible explanations for the decrease in fertility may be the physical conditions of the males (McDaniel, 1978), quality of semen and the selection for bigger BW presenting difficulties in transferring sperm into the oviduct and unsuccessful cloacal contact during mating (McGary *et al.,* 2003).

Female and male BB fertility is largely influenced by BW throughout their life. Van Krey & Siegel (1974) showed that females aged 8 weeks, selected for high BW had a lower plane of fertility during production. Leeson & Summers (2000b) explained that polycystic ovary syndrome in females, male leg problems and shrunken testes contribute to a large portion of fertility problems in obese BBs. The decrease in fertility is not only a result of excessive BW due to selection, but may be a result of the feeding programmes designed only to achieve a standard BW not including specific nutritional requirements of the birds (Walsh & Brake, 1997). Similar findings from Hocking & Duff (1989) were also reported, as well as that fertility of BB flocks has a tendency to decline as the age of the birds increase.

The turkey industry had immense problems associated with increased bird weight and drastic body conformation changes, where the birds were not able to copulate successfully and the use of AI was crucial in order for reproduction to be maintained (Ogasawara *et al.,* 1963). Artificial insemination has sparked the interest of many BB producers to guarantee higher fertilisation rates, however, time and funds may be limited. Alternatively, BB producers have had to apply numerous managerial strategies including strict nutritional schemes within the parent stock. These schemes aid to combat the negative effects on reproductive performance, such as reduced fertility associated with overweight birds (Romero-Sanchez *et al.,* 2007).

2.1 The Role of Nutrition in Broiler Breeder Fertility

Optimum reproductive performance of females and males is achieved by controlling BW of birds throughout the entire production cycle, providing nutritional requirements are met. Many experiments (Blair *et al.*, 1976; Cerolini *et al.*, 1995; Walsh & Brake, 1997, 1999; Zhang *et al.*, 1999) have reported a significant decline in eggs produced by females and lowered levels of fertility in males when metabolisable energy (ME) and CP requirements are not met.

Crude protein and energy (E) and their effects on fertility of both male and female BBs, has been researched with inconsistent findings. In some cases, researchers found that high CP intakes negatively affected spermatozoal characteristics (motility, viability, volume and concentration) (Hocking & Duff, 1989; Hocking, 1990; Hocking & Bernard, 1997). Wilson *et al.* (1987a; b), however, found no significant effect of CP intake on factors determining semen quality and fertility (semen volume, concentration, number of fertile eggs at candling at 18 days of incubation and activity of sperm).

2.2 Growth and Reproductive Performance in Breeder Males

Concern has been raised on the breeding performance regarding excessive BW of BBs, particularly frequency of mating and fertility, especially that of the males (Siegel & Dunnington, 1985). According to a commercial breeder company (Cobb-Vantress, 2008a); male BBs should never lose weight during the production cycle as this may result in lowered sperm quality and viability and they should also not weigh more than 5.5 kg. If heavier they become too heavy to mount the female and tend to become more square-like and unbalanced in shape as opposed to a more desired ‘V-like’ posture. This alteration causes mating efficiency to drop owing to unsuccessful copulations.

2.2.1 Nutritional Requirements for Growth and Reproductive Performance of Broiler Breeder Males

Nutritional requirements are defined as the nutritional 'needs' that an animal requires for growth, maintenance and reproduction. These requirements include CP, ME, AA's and minerals (NRC, 1994). Requirements for both male and female BBs, particularly males, are not clearly understood, in that results of studies conducted are conflicting and limited literature is available on specific requirements. The female requirements however are generally higher than those of the male, due to the nutrient requirements of egg production as opposed to spermatogenesis.

Sexual maturity and reproductive development of BBs depends on the nutrition provided during rearing. Early onset of sexual maturity and excessive ovarian follicular development has resulted from birds with higher BW and fat deposits (Hocking *et al.*, 1987; Yu *et al.*, 1992a, b, c). It is therefore crucial that feed restriction programmes are set in place early on in the rearing phase, to ensure reproductive performance is optimised (Lopez & Leeson, 1994).

The requirement for AA of BBs considers both requirements for maintenance and production. The efficiency of converting an AA (dietary nutrient) into a product (egg) is between 80-85 % efficient (McDonald & Morris, 1985), which must therefore be accounted for when formulating feed. Fisher *et al.* (1973) introduced the Reading Model, a factorial equation to determine the optimum intake for each AA for laying hens. The concept of this model can however be applied in other areas of nutrition in other species.

The objective of this study is to revise the effects of dietary protein on fertility of males; this chapter will focus mainly on the breeding period, as the management and feeding of the bird is likely to affect the performance of the male during production. Although the main focus for nutrient requirements is E and CP, it is important to remember that other nutrients (i.e. vitamins, minerals and other specific AAs) are also vital for the general well-being and productivity of the birds (Barber *et al.*, 2005).

2.2.1.1 Effects of Energy on Reproductive Parameters of Breeding Males

Energy requirements of BBs are more complex than those of CP requirements in that E is required for maintenance as well as for reproduction in the breeder (Emmans, 1994).

Attia *et al.* (1995) reported that when BB males received diets with low ME, the birds were able to maintain BW with little or no excess ME for BW gain. However, concern arose that the spermatozoal maturation process was altered, where motility was affected, resulting in fewer spermatozoa reaching the sperm storage tubules (SST's).

Brown & McCartney (1983) found that BW gain and mating performance was not satisfactory in males receiving restricted diets and stated that 1.92 MJ ME/bird/day was necessary to maintain BW and optimal breeding performance. Borges *et al.* (2006a) conducted an experiment where 5 treatment feeds, differing in ME (1.21, 1.30, 1.38, 1.47 and 1.55 MJ ME/bird/day) were fed to BB males. The results showed that BW (both fat and CP contents) of birds increased linearly as ME levels increased. Parameters of fertility (vigour, mobility, quality and quantity of semen) were assessed and found to be highest at 1.47 MJ ME/bird/day (confirmed by analysing the author's data), suggesting that this intake of ME is sufficient to meet the E requirements of the birds during breeding.

Although extensive research has been conducted to determine the optimum ME in a diet, findings are still inconsistent. Many of the researchers have not studied the same parameters (male and female fertility, BW and mating performance, egg fertility, semen quality, sexual activity) and in many experiments the birds were at different stages of production and often the ME content of the diet is reported but not the ME intake, making it difficult to compare (Brown & McCartney, 1983; Attia *et al.*, 1993; Attia *et al.*, 1995; Peak, 2001).

2.2.1.2 Effects of Crude Protein on Reproductive Parameters of Breeding Males

Body weight is predominantly controlled by varying the amount of feed consumed (feed restriction programmes). Findings by Hocking (1990), using naturally-mated BB males, found

that if BW was too much above or below target, suboptimum fertility resulted. Males that were too thin were not sexually capable, as testes development and semen production were compromised, whereas males with excessive BW were incapable of copulating and thus completing the mating process.

As a guideline, Cobb-Vantress (2008a) suggest that an adult male breeder can be kept in superior condition with 1.55 - 1.59 MJ ME/day and 20 – 21 g CP/bird/day. All amounts that are recommended for CP and ME are dependent on the condition and stage of the animal; feed allocation and nutrients can be increased or decreased accordingly.

Hocking & Bernard (1997) determined the effects that CP (110 or 160 g CP/ kg) and BW had on overall fertility of BB males of a particular strain. Birds were categorised into 3 BW groups and feed allocation was relative to BW. Lower fertility in one of the strains receiving 160 g CP/ kg was found, which was mainly coupled with larger/ heavier breast muscling, less frequent copulations and a high number of incomplete matings, possibly linked to unsuccessful cloacal contact. Fontana *et al.* (1990) found that when naturally-mated commercial BB males and females were fed separately, with males on a 12 % CP diet and females on a 14 % CP diet, egg fertility was significantly higher when compared to the control diet, where both males and females were fed 14% CP. No feed allocation or intake was reported, birds were fed to achieve BWs according to guidelines specified by the breeder. Semen concentration was the same in birds fed separately and birds fed from the same feeder, thus the increased egg fertility was attributed to the size and weight of the males, allowing them to copulate more successfully and not to semen concentration.

Borges *et al.* (2006b) conducted an experiment where 5 feeds, differing in CP (12.0, 14.2, 16.4, 18.6 and 20.8 g CP/bird/day) were fed to BB males between 27 and 61 weeks. Body weight of birds was not significantly affected by the different dietary treatments. Parameters of fertility (vigour, mobility, quality and quantity of semen) responded quadratically to dietary CP, where birds on extreme deficient or excess CP diets had decreased reproductive efficiency. The CP intake to optimise fertility was suggested to be approximately 16.9 g CP/bird/day (confirmed by analysis of the author’s data).

Wilson *et al.* (1987a) found no undesirable effects on BW, semen quality, testes weight or sexual maturity, of BB males on a restricted isocaloric diet (containing 12 to 14 % CP; 15.6 and 18.2 g CP/day respectively) from 20 - 52 weeks of age. A ration containing 16 % CP (20.8 g CP/day) was used as a control feed. It was found that males receiving the 15.6 g CP/day diet produced higher concentrations of semen than those receiving 18.2 or 20.8 g CP/day. Wilson *et al.* (1987b) also reported that BW, semen volume, concentration and sperm numbers were not affected by diets containing 12 or 15 % CP (14.4 and 18 g CP/day respectively). However, BB males receiving a 9 % CP (10.8 g CP/day) ration had significantly ($P<0.05$) lower BW as opposed to birds receiving 12 and 15 % CP.

The values given as a guideline by researchers are specific to the parameters and stages studied in the respective experiments. As with ME dietary inclusions, actual CP intakes are required to draw conclusions for optimal CP inclusions in a diet.

2.3 Feed Restriction Programmes and Regimens during Rearing and Reproductive Phases

There are many widely used feeding regimens in commercial production to control BW by restrictive methods (Mench, 2002; Cobb-Vantress, 2008a, b). Two commonly used quantitative restriction methods are skip-a-day (SAD) and limited-every-day (LED) feeding.

The SAD uses a balanced feed ration formulated to meet the requirements of the bird and is fed every alternate day. This practice has however been banned in the United Kingdom and many other countries in Europe (Hocking, 2004) for animal welfare reasons.

A preferred regimen, providing more uniformity for BW and muscular distribution is the LED-feeding regimen, where a limited amount of balanced feed is fed on a daily basis (Leeson, 2010). Not only is the LED programme better for the physical and physiological features (improved semen production, superior semen quality and larger testes weight) but also from a behavioural perspective, where the birds were found to be less aggressive.

The severity of feed restriction poses problems for both male and female BBs. If levels of feed intake are too low or too high, fertility, production and reproductive fitness may be compromised. Yu & Robinson (1992) found that egg quality (shell thickness and shell quality) and egg production were hindered under excessive restriction. Brown & McCartney (1986) performed an experiment in individually caged birds under varying levels of feed restriction (115, 100, 85, 70 and 55%) of the required intake. Results showed that semen quality (volume and sperm numbers per ejaculate) and testes weight were not altered by levels of 115, 100, 85, and 70% restriction, but adverse effects were noted in more severe (55%) dietary restriction.

Although it is essential to restrict feed in BBs, this may prevent the birds from getting crucial nutrients required for optimal growth, maintenance and reproduction (Blair *et al.,* 1976). Although difficult in practise, it is very important to consider requirements of the individual bird rather than the average bird as this may cause some birds (smaller conformations) to have an excess of nutrients and larger birds to have an under supply of nutrients (Blair *et al.,* 1976; Balnave *et al.,* 1978; Walsh & Brake, 1999). Flock uniformity is therefore crucial to ensure this.

An important factor when considering BW and nutrient requirements of the flock was highlighted by Romero–Sanchez *et al.* (2008) who found that it is essential to consider individual BW and nutrient intake. Larger birds have higher maintenance requirements and would most likely be subjected to nutrient deficiencies, because the flock is fed according to the nutrient requirements of the average bird. However, this method of feeding individual birds proves uneconomical in commercial situations. This deficiency ultimately results in lowered semen production and mating activity. The Reading Model (Fisher *et al.,* 1973) is therefore useful in production when considering the flock. The equation consists of 2 elements; the optimum intake of the average bird and the additional amount of AA worth feeding (beyond that of the average bird).

$$I= aE_{max} + bW + x(a^2\sigma_E{}^2 + b^2\sigma_W{}^2 + 2abr_{EW}\sigma_E\sigma_W)^{1/2}$$

$I= aE_{max} + bW$= Optimum intake for the average bird

$x\ (a^2\sigma_E{}^2 + b^2\sigma_W{}^2 + 2abr_{EW}\sigma_E\sigma_W)^{1/2}$= The additional amount of amino acid worth feeding

Where a= Requirement for egg output (mg AA/ g egg output (mg/ g))

E_{max}= potential mean egg output in flock (g/day)

b= Maintenance requirement (mg AA/ kg BW (mg/ kg W d))

W= Mean body weight (kg)

σ_E= standard deviation of egg output (E)

σ_W= standard deviation of body weight (W)

Although this equation applies to females- a similar concept would apply when considering male breeders. Males do not require the same parameters for egg production and require less AAs for production of spermatozoa and seminal fluid. However, should the requirements not be met, male productivity and reproductive efficiency would decrease and declines in fertility would be noted.

Although strict feeding programmes reduce the BW of the birds, it also subjects them to physiological stress as well as abnormal behaviour as a consequence of constantly being hungry (Mench & Falcone, 2000). This is particularly true in male birds in individual cages, used for AI, which is a possible result of boredom. Feed restriction causes problems of frustration such as aggression and pacing which may result in secondary stress problems, affecting welfare and fertility of the birds (Shea *et al.*, 1990; Kostal *et al.*, 1992; Hocking *et al.*, 2001). Kostal *et al.* (1992) reported that the level of feed restriction is positively correlated to the portrayal of abnormal behaviour. Males and females with excessively low BW's have been reported to eat eggs in attempt to gain nutrients (Hocking, 1990).

The feeding behaviour of broiler chickens has been significantly altered through the process of selection. A fowl in a normal environment would preferentially spend a large portion of the day foraging for food as opposed to having its food provided (Duncan & Hughes, 1972). However, BBs on a limited ration consume what is provided for them almost immediately, in less than 15 minutes in some instances as reported by Kostal *et al.* (1992) when observing BBs in individual cages. To compensate for chronic hunger, birds tend to over-drink (polydipsia) causing wet litter, thus water supply needs to be restricted to maintain litter quality (Kostal *et al.*, 1992; Hocking *et al.*, 2001).

Brake (2003) proposed that it is more beneficial to have a controlled feed allocation rather than a severe restriction of feed. This ensures that requirements of the bird are still met to a certain degree but the birds are not over-consuming, whereas severe restriction promotes elevated levels of nutritional deficiencies. Similarly, Romero-Sanchez *et al.* (2008) suggested that feeding programmes should be designed to maintain BW rather than focus on achieving a fixed, standard BW. Anonymous (2000) explained that restriction may be carried out by feeding mash as opposed to pellets, adjusting lighting programmes so as to reduce light exposure, controlling the amount of feed per bird per day and restricting water consumption to a certain degree.

Romero-Sanchez *et al.* (2008) highlighted the differences between feeding constant daily amounts and increasing increments to BBs throughout the production cycle. Requirements of the larger birds with the greatest genetic potential were met by the increased feed allocation and allowed the birds with the largest BW's to continue to mate and produce progeny. Providing adequate nutrients to birds until peak production to sustain active mating and sexual behaviour does not only result in higher levels of fertility in the flock but also boosts the overall potential offspring for growth and feed efficiency (Romero- Sanchez *et al.,* 2008).

2.3.1. Growth control during Rearing

A number of studies have been conducted to determine the effects of feed restriction in early development of chicks. A suggestion made by many researchers (Harms *et al*, 1984; Crouch *et al.,* 2002; Mench, 2002) is that BW control in immature birds is a major aspect concerning subsequent fertility. It is important to note that birds being reared on a restrictive feeding programme should not lose BW and condition but rather maintain BW within breed target to ensure reproductive efficiency later on (Harms *et al*, 1984; Crouch *et al.,* 2002). However, the rate of BW gain fluctuates slightly depending on organ development and degree of maturity of the bird.

Ferket & Moran (1986) conducted an experiment to determine the effects of CP on fertility of turkey hens fed at early stages of development (day old to 31 weeks). They found that birds fed low CP diets during rearing had improved levels of fertility at the end of production.

Sexton & Renden (1988) conducted an experiment to determine whether feeding regimen had an effect on body composition, gastrointestinal tract size, and semen quality and found that birds fed the SAD regimen had delayed sexual maturity, sometimes up to 10 days, than birds fed on a daily basis. Zhang *et al.* (1999) conducted an experiment to determine the effects of CP during the rearing period on BW and semen production of BB males. Two strains of birds were fed either 12 or 16 % isocaloric diets on the SAD regimen (no feed intake was reported). Body weights between 4 - 28 weeks were found to be higher in birds fed 16 % CP than 12% CP. The birds receiving less CP (12 %) had higher semen volume than birds on the 16% CP diet. A negative correlation was found between BW and semen volume.

2.3.2 Body Weight Control during Breeding

Within a restricted feeding regimen, there are 4 important phases. Initial intake, which is designed according to rearing BW, to ensure requirements for growth are met; feeding ahead of peak production (lead- feeding), to ensure body reserves are maintained for production; maximum intake, to provide sufficient nutrients at peak production for continued development of bird weight and production and withdrawal of feed after peak production, to control BW and prevent obesity (Ross Breeders, Aviagen, 2007). It is important to note that during the different phases of growth and production the requirements of the birds change constantly. Once the birds have reached sexual maturity, controlling BW to prevent obesity and related reproductive problems is essential.

Although the time to slaughter of a broiler has been greatly reduced and the yield of breast-meat increased, the problem of fertility lies at the bird itself. It seems that it is BW that affects the productive and reproductive fitness of the birds (Wilson & Harms, 1986; Cobb- Vantress, 2008b). Birds that are obese or excessively overweight are not able to copulate successfully, due

to lack of cloacal contact during mating. The actual body conformations and size of the bird play a role in this inability to copulate successfully.

Siegel & Dunnington (1985) stated that it is specifically the male BW that directly affects reproductive fitness in BBs due to heavier body conformations. However, opposing this statement, McGary *et al.* (2003) found no correlation between fertility and male BW or sperm penetration of the perivitelline membrane, as a measure of fertility in 2 different strains of BB, reared in blackout housing from week 7 to 21 on a restricted diet (undisclosed information). Hocking & Bernard (1997) suggested that male BBs should be fed to allow for small, yet steady increases in BW to ensure that fertility, health and fitness are maintained during breeding. Hocking (1990) reported that males larger than 5 kg were comparatively infertile and dominated smaller males preventing them from mating or obtaining feed, rendering them unable to maintain health and reproductive fitness.

2.4 Assessment of Fertility in Broiler Breeder Flocks

Many definitions of fertility have been put forward. Pearl (1917) defines fertility as, "the total net reproductive capacity of pairs of organisms, male and female, as indicated by their ability to produce viable individual offspring." For comparative reasons and to eliminate confusion Wishart (1985) stated, "Fertilising ability appears to be best described as the numbers of spermatozoa required to give a particular level of fertility, rather than the fertility obtained by inseminating a particular number of spermatozoa". Brillard (1993) explained fertility as "The number of days during which a female lays fertile eggs after a single copulation or artificial insemination". Wishart *et al.* (2001) described individual males and females as fertile when they are 'capable of breeding' resulting in a 'fertile' egg being produced, with the 'capability of hatching'. Successful fertilisation is dependent on a number of factors which contribute to the process before, during and after the course of mating or AI.

One of the problems in attempting to determine the effects of nutrition on fertility is the measurement of fertility and the need to separate the male and female contribution to egg fertility. This section will look at some measures of fertility in BBs.

Avian species have a reproductive advantage in that after successful copulation or AI, the females have the ability to store sperm in the utero-vaginal junction (UVJ), within the discrete SST's (Bakst, 1981). There is no need for precise synchronisation of ovulation and copulation and the presence of a male is not continually required in order to produce fertile eggs as only fecund sperm are stored in the SST's (Bakst *et al.,* 1994). Sperm rapidly fill the SST's, as seen in hens that were killed 10 minutes after a single copulation or insemination already had abundant spermatozoa residing in the UVJ (Bobr *et al.,* 1964). The filling of SST's is slower in turkeys (2 days) as compared to chickens (1 day), however the release of the sperm is more rapid in chickens resulting in the fertile period being less prolonged (Brillard, 1993). At the time of release the spermatozoa ascend to the site of fertilisation in the infundibulum (Bakst, 1981). Contact is made between the blastodisc and the spermatozoon, through acrosomal activity, and the egg is fertilised.

It was previously reported that the 'sperm-nests' in the infundibular region were storage sites for sperm, however, after various experiments to determine the distribution of sperm using different AI methods it was concluded that the SST's were in fact only in the UVJ region, despite an increased concentration of sperm in the infundibular region (Van Krey *et al.,* 1966). All sperm found in the oviduct are perfectly normal and viable, which suggests that the oviductal glands possess some mechanism for barring abnormal, dead or contaminated sperm as reported by Bobr *et al.* (1964), which allows selection of spermatozoa by the female (Bakst, 1989). Brillard (1993) explained that the maximum efficiency of sperm storage, and thus the duration of fertility, depends on the numbers of sperm residing in the SST's after AI or copulation. According to Wishart (1987) the duration of fertility is, "the number of days until an egg with a 50:50 chance of being fertile is laid." Wishart (1987) and Wishart & Staines (1999) found that the number of spermatozoa after copulation or AI in the first laid fertile egg indicates the length of the fertile period, which decreases linearly as the number of spermatozoa in the SST's decrease. In the experiments performed by Taneja & Gowe (1961), a hen was not pronounced infertile unless she had laid six successive infertile eggs after a fourteen-day period after insemination. Aging hens tend to show a decline in fertility even after repeated inseminations. In turkeys, it was first believed that decreased fertility was due to the reduced capacity of the SST's to store spermatozoa (Wishart, 1987). However, this has been refuted. Twenty four hours post-

insemination, old hens had 1.831 x 10^3 spermatozoa in the SST's and young hens had 1.833 x 10^3 (Wishart, 1987), a notably small difference. Brillard (1993) reported that the rate of sperm release was double in old hens in comparison to that of young hens, and it was concluded that a decline in fertility as age increases is caused by the increased rate of sperm release from the SST's rather than the SST capacity.

Following AI or copulation most spermatozoa are released from the vagina and less than 2% of an inseminated dose of 100 to 200 million spermatozoa are found in the UVJ SST's (Brillard, 1993). Timing of insemination, with regard to the hen's cycle and the receptivity of the hen itself is important when artificially inseminating, whereas with natural mating or copulation, the frequent copulations with the male ensure that the SST's are filled regularly. Chickens inseminated a few hours before or after oviposition remain sub-fertile, according to Brillard *et al.* (1987). This may be due to the hen's inability to store adequate numbers of sperm to fertilise the egg (Brillard, 1993). Moore & Byerly (1942), Hammond *et al.* (1971) and Lodge *et al.* (1971) noted that a maximum fertility was attained between the second and fourth day following insemination, although fertile eggs were laid earlier, but fertility decreased sharply by day six following insemination. Bramwell *et al.* (1995) explained that eggs laid on the first day post-insemination would already have had the additional protective outer covering of the OPVL around the yolk and germinal disc, preventing the penetration of IPVL resulting in a decreased fertility of eggs. Contrasting the idea proposed by Bramwell *et al.* (1995), Burrows & Quinn (1938) assumed that the day following actual introduction of the spermatozoa into the oviduct is the day of conception. Bakst (1988) noted that high fertility rates were obtained in turkeys when the first insemination was performed before the onset of egg production. However, in chickens this is not possible because the eversion of the cloaca can only be performed after the first egg has been laid. The increased efficiency in turkeys was due to the increased numbers of spermatozoa in the SST's, in the absence of the contractions or cyclic oviductal secretions presented once egg production commences.

In order to determine the male role in fertility of BBs Wishart (1987) established a non-invasive method of determining the density of spermatozoa embedded in the OPVL of eggs. Brillard & Bakst (1990) stated that this technique is reliable for the evaluation of semen quality and the

capacity of the SST's. Wishart (1997) proposed that quantification of the $OPVL_{sperm}/mm^2$ membrane can be used to predict the fertility status of the egg.

2.4.1 Cockerel Fertility Affected by Body Weight

The level of fertility in male BB is portrayed through sexual behaviour, i.e. libido and courting, where Siegel & Dunnington (1985) found that males with high BW's courted 3 times less than those of lower BW's. Physiological function and physical characteristics also play a vital role in the level of fertility portrayed by the male, as well as measures of semen quality.

Hocking & Duff (1989) showed that skeletal and leg dimensions were a factor contributing to reduced reproductive viability, resulting from altered conformations due to selection in BBs. The reasoning was that the birds were not able to physically support their bodies resulting in unsuccessful copulations. Skeletal alterations may hinder semen transfer by limiting cloacal contact during copulation. In an experiment by Bilcik *et al.* (2005) to determine the reproductive success and effects of body condition on fertility, a positive relationship between frequency of matings without cloacal contact and BW was found, resulting in ineffective fertilisation from such matings. Therefore, a higher frequency of mating does not necessarily indicate higher fertility because not all observed matings are successful.

McGary *et al.* (2003) determined the correlation between physical traits and fertility in BB males. One of the parameters studied was pelvic conformation. It was found that a strain that had been genetically selected for yield had a significantly larger dorsal pelvic width which was associated with lower fertility, when compared to another strain. A likely explanation is that during the selection for higher yield, the skeletal structure of the breed was slightly altered to accommodate the larger breast conformation. The widening of the pelvic width may have hindered cloacal contact during mating.

2.4.2 Reproductive Physiology of the Male Broiler Breeder

Male avian reproductive physiology differs to a large degree from most mammals in that the testes are situated internally and the spermatozoa are able to remain viable at body temperature which is detrimental to the survival of sperm in other species. Both testes are functional in the male bird as opposed to only the left functional ovary and oviduct in the female (Etches, 1996).

The epididymus and the vas deferens convey the sperm away from the testes, allowing for expulsion of the stored sperm from the vas deferens at ejaculation (Lake & Stewart, 1978). The protruding phallic folds of the ventral cloaca are filled with lymphatic fluid and thus allow the semen to flow through the depression (Howarth, 1995; Etches, 1996). According to Garner & Hafez (1987) there are approximately 0.06 - 3.5 billion sperm/ejaculate and between 3000 - 7000 million sperm/ ml.

Howarth (1995) stated that sexual maturity in cockerels is the age of first appearance of spermatozoa in a sample of ejaculate, which usually occurs between 16 and 24 weeks of age. Testes size in BB males is not constant, as they generally become larger once sexual activity commences and regress towards the end of the production cycle. Amann (1999) found a positive relationship between testis size and sperm production. Fontana *et al.* (1990) and Leeson & Summers (2000a) found a positive correlation between body size and testes size during breeding, indicating that males that loose body condition during breeding may begin testis regression.

Secondary sexual characteristics such as comb size, colour and area are non-invasive measurements of fertility and testes size (Zuk *et al.,* 1990a; McGary *et al.,* 2002; Tyler & Gous, 2008). Burrows & Titus (1939) first discovered that larger combs indicated higher semen production, which was also confirmed by Zuk *et al.* (1995) and McGary *et al.* (2002), where comb area was an indicator of higher fertility and larger testes (Pizzari *et al.,* 2004) and thus amplified semen production.

2.4.3 Assessment of Semen Quality of Broiler Breeder Males

Seminal fluid comprises both seminal plasma and spermatozoa. Evaluation of a semen sample is important when considering semen quality and capability. Both visual and microscopic evaluation of a semen sample can be done to determine quality and viability of the spermatozoa in the sample.

Among the various methods of semen analysis, the more commonly used methods include visual assessment (where discolouration of the pearly-white sample may be indicative of poor quality (Etches, 1996)), semen concentration (the total number of spermatozoa in a sample of ejaculate (Etches, 1996)), percentage of live and dead sperm (the number of live and dead spermatozoa in a sample), morphology (the size and shape of the spermatozoa), mobility (the net forward movement of a sperm population penetrating a density gradient (Froman & Feltmann, 1998)) and motility (the visual assessment of the movement of the semen sample under light microscopy (Froman, 2007)).

Assessment of sperm quality can also be assessed through egg fertility using macro- and microscopic assessment.

2.5 Assessment of Egg Fertility

Fertility of eggs does not only rely on the outer conformation/ physical characteristics of the bird but also the environment to which the ovum, spermatozoa and fertilised eggs are subjected to (Brake, 1997). The notable decline in fertility and hatchability of BB over the years has sparked debate within the BB industry (Aviagen Limited, 2001; Cobb-Vantress, 2008a; Waldroup, 2011).

Determination of egg fertility prior to hatch can be assessed by macroscopic and *in vitro* examination of a sample of eggs to indicate flock fertility and give an indication of the potential for hatch.

2.5.1 Macroscopic Examination

Determination of fertile eggs at an early stage of production is very important in poultry enterprises (Liptói *et al.*, 2004) as it gives an indication of productivity and reproductive success of the flock. Kosin (1945) stated that by distinguishing between the spherical appearance of the fertile blastoderm and the condensed knot of opaque material and crater-like lacunae in an infertile egg, fertility can be assessed in the fresh, unincubated egg. However, incorrect predictions of fertility may result from mistaken recognition of the fertile blastoderm (Wishart 1997).

Van Krey *et al.* (1966) determined fertility by means of macroscopic examination of blastodiscs after an incubation period of 24 hours, when they were assessed for evidence of embryonic development. However, this method does not recognise early embryonic mortality, which could arise as a result of incorrect handling (before or during incubation), flock health, incorrect incubation positioning, incorrect incubation settings (humidity and temperature), time kept in storage before being placed in the incubator and chemical exposure to eggs (Kosin, 1964; Wilson, 1991; Wilson, 2007).

Although useful in poultry enterprises, macroscopic assessment of fertility in eggs is destructive and therefore only a sample of eggs can be tested which does not always give an accurate indication of overall population.

A more common method, candling, first described by King (1936) is used to detect developing embryos during the first day of incubation. After 22 hours, the eggs are positioned for 2 hours to allow for orientation of the blastodisc to the uppermost portion of the shell and each egg is then presented to the candling-lamp without rotation and a developing blastodisc can be seen through the translucent shell as a dark spot.

2.5.2 *In Vitro* Examination

Another, more recent, method of determining fertility is the examination of both inner and outer perivitelline layers of the egg. However, both methods of IPVL and OPVL examination are destructive, where eggs are broken during different stages of development, and therefore only a sample of the represented flock can be used.

According to Brillard & Bakst (1990), Brillard & Antoine (1990) and Wishart & Staines (1999), the number of sperm interacting with the perivitelline layer is highly correlated with the insemination dose, number of spermatozoa present in the SST as well as the probability of fertilisation.

Once the spermatozoa are released from the SST's they bind to the ovum in the infundibulum within 10 to 15 minutes of ovulation. This is to ensure attachment to the ovum prior to the OPVL being deposited, which is soon after ovulation (Wishart & Staines, 1999). Post-attachment, and prior to the secretion of the OPVL, the spermatozoa undergo an enzymatic reaction, where acrosomal proteases hydrolyse a small hole in the layers of the IPVL, which a single spermatozoon will successfully enter and fertilise the ovum (Wishart *et al.,* 2001), although, depending on the numbers of spermatozoa trying to fertilise the egg, many points of hydrolysis may occur. The spermatozoa that have not successfully fertilised the egg become trapped in the fibrils of the newly formed OPVL (Wishart *et al.,* 2001). Bramwell *et al.* (1995) and Bramwell (2007) explained the final stage of fertilisation as the binding and penetration of spermatozoa to the egg, prior to the addition of the OPVL.

2.5.2.1 Examination of the Inner Perivitelline Layer

Sperm penetration (determined by the number of holes in the IPVL) is probably the best way to detect fertility as it is more sensitive and reflects small changes in spermatozoon activity at the site of fertilisation and detects early problems in flock fertility which can be corrected (Bramwell *et al.,* 1995). As the spermatozoa undergo the acrosomal reaction, holes appear in the IPVL (Bakst & Howarth, 1977). The holes hydrolysed in the IPVL by the acrosomal proteases of the

spermatozoa are notably higher around the germinal disc (Wishart & Staines, 1999), and there may be as many as 1000 IPVL holes, although as little as 6 holes are required to produce a fertile egg (Wishart, 1997); the probability of fertilisation is increased as the number of IPVL holes increase (Bramwell *et al.*, 1995). Bramwell *et al.* (1995) stated that the more sperm available for penetration around the germinal disc, the higher the probability of syngamy. Albeit, numerous experiments (Wishart, 1987; Bramwell *et al.,* 1995; Wishart & Staines, 1999), suggested that there is a minimum number of sperm required for fertilisation to be successful.

The interval for interaction between the spermatozoa and the IPVL is estimated at around 8 - 10 minutes, and ceases when the OPVL is secreted because it prevents any further spermatozoal interaction with the IPVL.

2.5.2.2. Examination of the Outer Perivitelline Layer

Minutes after fertilisation of the ovum, the second layer of membrane (OPVL) is secreted, trapping excess spermatozoa in the protein fibrils, which are visible but have not necessarily penetrated the IPVL (Wishart *et al.*, 2001). This layer acts as a barrier and retains the integrity of the ovum (Bakst & Howarth, 1977). Brillard (1993) also showed that the number of spermatozoa in the OPVL correlated with the number of spermatozoa extracted from the SST's and therefore these tests assessing the egg and sperm interaction are useful to predict fertility. However, the technique of OPVL examination does not take into account the presence of impaired spermatozoa in the layer, which are not available or capable of fertilising the egg, resulting in decreased numbers of sperm penetration holes (Bramwell *et al.*, 1995).

There is a positive relationship between the number of sperm trapped in the OPVL and probability of fertility. This, however, does not indicate the number of bound or penetrated sperm, which requires further examination of the IPVL to determine fertilisation, visualised as microscopic holes in the IPVL (Wishart, 1987). The method of examination of OPVL can be used as a measure of semen quality with *in vitro* tests of binding (Hazary *et al.*, 2001) or as an assessment of fertility in broken eggs (Hazary *et al.*, 2001).

Estimating the number of spermatozoa trapped in the OPVL of unincubated eggs is one of the methods used to determine the probability of fertilisation as well as the length of the fertile period (Wishart, 1997). The relationship between $OPVL_{sperm}$ and fertility has been found to be positive, but reaches a plateau, at approximately 50 x 10^6 sperm (Wishart & Staines, 1999).

According to Wishart (1987), for an egg to be fertile there should be > 0.3 to 0.4 OPVL sperm/mm^2. A 50 % probability of an egg being fertile is associated with 0.1 sperm/mm^2 OPVL and eggs with < 0.05 sperm/mm^2 OPVL are infertile (Wishart, 1997). Wishart (1997) found that the number of sperm in the OPVL after a series of natural matings was double that in the OPVL after AI. This may be due to the increased frequency of natural mating filling the SST's constantly, as opposed to a single filling after AI (Wishart *et al.*, 2001). An important aspect to remember when performing AI is that the number of sperm in the OPVL decreases logarithmically in successively laid eggs, at a loss of approximately 30 % per day (Bramwell *et al.*, 1995).

2.6 Conclusion

Excessive growth rates, distorted muscular distribution and the inability of BBs to control feed intake has caused a number of complications in the industry regarding fertility and successful copulations. Although many scientists have researched the effects of feeding regimens and dietary CP intakes, there are many contradictory findings and gaps in the literature and because genetic selection is continuous, it is likely that requirements change with progress in selection. Early assessment of fertility through micro- and macroscopic evaluation is crucial for optimal flock productivity and viability.

The aim of this dissertation is to determine more accurately the response in male fertility to CP intakes in the diet to maximise male productivity.

CHAPTER 3

THE EFFECT OF DIETARY CRUDE PROTEIN ON FERTILITY OF BROILER BREEDER MALES

3.1 Introduction

Many factors influence and contribute to the fertility and hatchability of eggs. Hen fertility is a straightforward assessment calculated by the number of fertile eggs produced. However, male fertility is a more complex procedure where a number of aspects need to be considered and requires more intensive measures (Hays, 2007). In modern-day broilers and BBs, a great concern regarding fertility is BW (Lake, 1989). Stringent feeding regimens in both female and male BBs to control BW are set in place to achieve optimum reproductive performance (Emmerson, 1997). These regimens focus primarily on reducing levels of CP and reducing actual feed intake, forcibly keeping BWs below optimum to obtain favourable production and reproductive qualities in BBs.

Various levels of CP included in a diet are found to have negative effects on BW, fertility and spermatozoal characteristics (Hocking, 1990; Hocking & Bernard, 1997), all of which relate to overall productivity of the flock. It is important to determine the optimal inclusion of CP in a diet to deter from negative aspects caused by inadequate or excess CP. Inconsistent findings amongst researchers has resulted in a number of experiments to find the optimum CP intake.

Natural mating consists of a number of behavioural courtship displays before mating is complete (wing dropping, shuffling, side-stepping, dancing in a circle and scratching) a term described by Kruijt (1962) as ‘waltzing’. Once mounted, the rooster treads quickly and drops his tail, where cloacal contact is established (Kruijt, 1962). Although it is easier to measure fertility of males in individual cages, this removes the behavioural aspects of mating. It is thus important to determine whether males in individual cages will reflect the same results in research as those

under commercial (natural mating) conditions. From a research point of view, it is important to note the differences in natural-mated flocks and those requiring AI. Koohpar *et al.* (2010), compared natural mating and AI in the Mazandran Native hen and found no significant difference between hatching traits of these 2 methods. However, this has not been evaluated in commercial meat-type hybrids that are well known for displaying a lower fertility.

The objective of this experiment, therefore, was to investigate the effects of dietary CP on fertility of BB males observed through hatchability and examination of the OPVL of the egg, and to compare the response to CP of males in individual cages to those in a natural mating flock.

3.2 Materials and Methods

Thirty Cobb 500 BB males, at 29 weeks of age, were randomly allocated to 6 open-sided, natural daylight floor pens (5 per pen) and 50 females of the same age and strain were also placed in each pen, allowing for natural mating. Artificial lighting (13L: 11D) was provided and natural photoperiod was <13 hours. The breeder males were fed a commercial broiler breeder pellet up to 28 weeks of age. Thereafter, each group of 5 males was randomly assigned to an experimental treatment feed and fed for a further 34 weeks. The females continued to consume the commercial breeder pellet until the end of the experiment.

Seventy two Cobb 500 BB males of the same stock at 29 weeks of age were placed into individual cages, in ventilated, light tight rooms. There were 12 cages in each of the 6 rooms, with 2 males per treatment in each room, resulting in 12 males per treatment. These birds were also provided with 13L: 11D.

Two basal feeds, a balanced summit feed and a non-protein diet (N-free diet) were formulated (Table 1) using the AA requirements proposed by Gous & Nonis (2010). The balanced feed was formulated to provide balanced levels of AA’s in the diet, where all AA’s were formulated to provide 1,2 x the requirement.

Table 1. *Ingredient composition of the balanced summit Feed and N- free diet;*

Ingredients		**Balanced feed**	**N-Free diet**
Yellow maize fine	g/kg	624	-
Soybean 50	g/kg	257	-
Filler	g/kg	-	534
Sand	g/kg	-	267
Sunflower Husks	g/kg	-	267
Oil - sunflower	g/kg	-	250
Sugar	g/kg	-	125
Limestone	g/kg	64.3	64.7
Wheat bran	g/kg	36.4	-
Sodium bicarbonate	g/kg	5.80	1.40
Vit+min premix	g/kg	1.50	1.50
Salt	g/kg	1.10	3.60
Potassium hydroxide	g/kg	-	9.70
Monocalcium phosphate	g/kg	6.00	10.0
L-lysine HCl	g/kg	1.50	-
DL methionine	g/kg	1.50	-
Tryptophan	g/kg	0.30	-

The two isocaloric (11.4 MJ/ kg) basal feeds were blended to provide 6 experimental feeds (Table 2) with an analysed range in protein content from 86.8 to 168 g CP/kg (Table 3).

Table 2. *Blending proportions and crude protein intake (CP) of the experimental treatments;*

Treatment	Balanced feed (%)	N-Free diet (%)	CP Intake (g/bird/day)
1	100	0	20.1
2	89	11	19.3
3	78	22	17.8
4	67	33	14.2
5	56	44	12.4
6	44	56	10.4

Table 3. *Analysed nutrient composition of the six dietary treatments;*

Nutrients	Units	1	2	3	4	5	6
AMEn_adult	MJ/ kg	11.5	11.3	11.5	10.6	10.7	9.94
Crude protein	g/kg	168	160	148	118	104	86.8
Lysine	g/kg	8.40	8.00	7.50	5.60	4.70	2.90
Methionine	g/kg	3.80	3.30	3.10	2.20	1.30	1.30
Threonine	g/kg	4.80	4.40	3.90	3.20	2.60	1.80
Arginine	g/kg	9.30	8.90	7.70	5.90	5.00	3.80
Isoleucine	g/kg	6.20	6.30	5.30	4.20	3.40	2.50
Leucine	g/kg	15.8	6.80	13.7	10.6	9.90	6.60
Histidine	g/kg	3.40	3.30	2.70	1.90	1.70	1.30
Phenylalanine	g/kg	6.70	6.80	5.90	4.50	4.00	2.90
Tyrosine	g/kg	4.40	4.40	3.70	2.80	2.10	1.60
Valine	g/kg	7.00	7.30	5.90	4.60	4.80	3.10

After mixing, the feeds were sampled and analysed for nutrient composition (Table 3). Methods used to determine nutrient compositions of experimental feeds were from McNab & Fisher (1984) for AMEn (MJ/ kg), the Association of Official Analytical Chemists (AOAC) Official Method 990.03 (1995) for crude protein and Moore & Stein (1984) for AAs.

All females received 160 g of a commercial feed per day (containing 150 g fat/ kg and 135 g CP/ kg) and males received 120 g of assigned treatment feed per day (Cobb-Vantress, 2005). The dietary treatments were fed to males both in natural mating pens via separate-sex feeders and to birds caged in individual cages via individual feeder troughs. Birds in each pen had *ad libitum* access to water until 15:00 daily. The feed allocation throughout the experiment was kept constant. The BB males were weighed individually at the beginning of the experiment and once a month thereafter.

Artificial insemination, a technique described by Burrows & Quinn (1937) is still used to date. The ‘abdominal massage method’ of semen collection is a non-invasive method, which stimulates ejaculation of semen by roosters used for insemination. The bird is held by a technician, with the wings restrained and the legs are held firmly, while another trained technician massages the back/tail area and gently applies pressure to the cloaca to stimulate semen production (Bakst & Brillard, 1995). An erection of the phallus results in the expression

of semen, which is collected into a clean container (Lake & Stewart, 1978). Initially semen training occurred 3 times per week to allow the male to become accustomed to the procedure and to obtain good quality semen (Lake & Stewart, 1978). When no sample was obtained, the procedure was not repeated immediately, only again on the alternative day (Bakst & Brillard, 1995).

The males in individual cages were not massaged for 2 days before collection for insemination, to ensure maximum semen yield. Care was taken during collection to prevent faecal contamination and collection of the clear watery fluid indicating absence of spermatozoa (Bakst & Brillard, 1995). Semen from males from each treatment that consistently produced was pooled and used to artificially inseminate a maximum of 5 females per treatment.

A sample of 50 µl of the undiluted pooled sample from each treatment was added to 8 ml of eosin and nigrosin solution, and the concentration determined with the use of a haemocytometer. An assumed value of 90% live sperm was used to calculate the dilution required to inseminate a fixed concentration of 60 million sperm/50 µl per insemination. The diluent used was Tyrodes solution (Allen & Grigg, 1957) (Appendix A).

Within an hour of semen collection, the hens were inseminated. They were held by a technician and the cloaca gently everted to expose the entrance to the reproductive tract. Fifty µl of diluted sperm was inserted into the vagina with the use of a micro-pipette. Insemination was performed in the late afternoon to ensure that oviposition had occurred more than 2 h previously which would otherwise negatively influence fertility (Brillard & Bakst, 1990). The day on which insemination was performed was referred to as day 0 (D0). Eggs from inseminated birds were collected on D2, D3 and D4 post AI.

Fertility was determined by hatch percentage of number of eggs set (Table 4) and by the examination of $OPVL_{sperm}$ of oviposited eggs (n= 30 for each treatment) at 31, 45 and 59 weeks of age in the natural mating males and at 45 and 59 weeks in the males in individual cages after semen collection and AI. Males in individual cages were not assessed at 31 weeks as semen training did not yield enough semen for AI at this age. No eggs from AI birds were used for

$OPVL_{sperm}$ examination at any age period, as the sample size of eggs was too small and thus all eggs were incubated to determine fertility and hatchability.

At each assessment age, eggs from each natural mating pen and inseminated birds were collected 4 times a day over a 5-day period, and settable eggs were marked and stored for up to 1 week at 13 ^{o}C, before placing trays of each treatment in an incubator (Table 4). After the incubation period of 21 days the percentage hatch was recorded, and break out of hatch debris was performed to determine the number of infertile eggs (no embryonic development) or stage of embryo death. Any presence of blood vesicles, a fertilised blastodisc and an embryo were recorded as fertile, and stage of embryo death recorded accordingly. An egg was pronounced infertile when none of the above parameters were visible.

Table 4. *The total number of eggs set for each treatment at different ages*

Protein Intake (g CP/bird/day)	Artificial Insemination		Natural-Mating		
	Number of Eggs Set		Number of Eggs Set		
	45 weeks	59 weeks	31 weeks	45 weeks	59 weeks
10.4	6	12	150	120	105
12.4	5	12	150	120	105
14.2	6	12	150	120	114
17.8	8	12	150	120	105
19.3	8	12	150	120	113
20.1	9	12	150	120	114

Remaining eggs (n= 30) from naturally mated birds from each treatment were used to examine the number of sperm trapped in the OPVL using the method described by Wishart (1987) to determine $OPVL_{sperm}/mm^2$ at 31, 45 and 59 weeks. The eggs were cracked open and the yolks separated from the albumen. Excess albumen was removed by rolling the yolk on paper towel. The germinal disk was located, and a square of approximately 1 cm^2 of the perivitelline membrane was cut around the germinal disk and rinsed 3 times in Dulbecco's phosphate buffered saline (PBS) to remove surplus yolk before it was stretched out on a glass microscope slide. The membrane was stained with 1µg/ml solution of diamidinophenylindole (DAPI) fluorescent dye and covered with a cover slip (Wishart, 1987). The slides were placed in a light

tight container, to prevent the dye from denaturing, and examined on the day of preparation. The slides were examined under fluorescent microscopy and sperm embedded in the OPVL were counted and totalled in 20 randomly chosen fields of view, under 40 x magnification. These numbers were then expressed as sperm/mm^2 of membrane ($OPVL_{sperm}/mm^2$). These results were used to assign the probability of each egg being fertile, based on Wishart (1997) and Brillard & Antoine (1990). Wishart (1997) found that an egg requires 3 or more spermatozoa /mm^2 to have a 94% probability of fertility. Brillard & Antoine (1990) in a similar experiment suggested that an egg only requires 0.43 $OPVL_{sperm}/mm^2$ membrane to have a 100% probability of being fertile.

3.3 Statistical Analysis

As this experiment was performed to determine the response in fertility to dietary CP intake, there were no replications, because it was deemed more important to have more points on the regression to determine an accurate response than to be able to determine differences between treatments. A similar design was followed in female breeders by Lewis *et al,* (2008). However, experiments run without replications have large room for experimental error, which can be avoided through replication.

Mean BWs of the males in natural-mating pens were subjected to linear regression and mean BWs of the males in individual cages were subjected to polynomial regression (as there was no significant linear response in this case) to determine the relationship between BW, age and crude protein intake.

Regression analyses were performed to determine the response in hatchability of both individually-caged and naturally-mated birds over the range of CP intakes. No ANOVA was performed for hatchability due to lack of replications, however the data were reported. No conclusions were drawn from these values due to lack of statistical analysis.

The $OPVL_{sperm}/mm^2$ examination of eggs from natural-mating hens over the range of CP intakes was not normally distributed, hence the minimum and maximum values were reported rather than the standard deviation, and a log transformation was performed prior to regression analysis.

Because there were several observations without any sperm (i.e. values of 0), a constant of 0.5 (chosen as it can be log transformed, but is small enough not to influence the remaining data) was added to each measurement. Analysis of $OPVL_{sperm}/mm^2$ is a preferred method of determining a response in fertility, as it provides continuous data rather than the comparison of binomial (fertile versus infertile) data as provided by the Brillard and Antoine (1990) and Wishart (1997) methods.

The percentage of eggs predicted to be fertile according to the calculations of Brillard & Antoine (1990) and Wishart (1997) over the range of CP intakes were analysed by simple linear regression with age as a group, and also over the range of ages with CP intake as a group. Genstat 11th Edition (2008) was used for the statistical analyses.

3.4 Results and Discussion

3.4.1 Body Weight

Results from this study show that birds that consumed less protein lost weight by the end of the experimental period whilst those that consumed more protein gained weight, regardless of whether they were individually caged or naturally mating (Table 5). These findings concur with findings of many researchers (Cave, 1984; Wilson *et al.*, 1987b; Emmerson, 1997; Zhang *et al.*, 1999; Ross 2007; Cobb- Vantress 2008a; Romero- Sanchez *et al.*, 2008) where BW has been restricted by CP.

Table 5. *Initial, final and body weight (BW) gains (± s.e.m) of males receiving different crude protein intakes (g CP/bird/day) in individual cages and natural mating pens.*

Protein intake (g CP/bird/day)	Body Weight (kg)					
	Individual cages			Natural- mating pens		
	Initial BW	Final BW	Weight gain	Initial BW	Final BW	Weight gain
10.4	3.84 ± 0.08	3.57 ± 0.13	-0.27 ± 0.13	4.28 ± 0.12	3.65 ± 0.16	-0.63 ± 0.23
12.4	4.10 ± 0.10	3.77 ± 0.19	-0.33 ± 0.19	4.34 ± 0.14	3.72 ± 0.23	-0.63 ± 0.26
14.2	4.10 ± 0.08	3.96 ± 0.13	-0.14 ± 0.15	4.00 ± 0.04	4.31 ± 0.18	0.30 ± 0.20
17.8	3.87 ± 0.12	4.56 ± 0.08	0.69 ± 0.12	4.47 ± 0.14	4.83 ± 0.26	0.36 ± 0.25
19.3	4.04 ± 0.07	4.73 ± 0.18	0.70 ± 0.17	4.12 ± 0.16	4.44 ± 0.26	0.33 ± 0.34
20.1	4.09 ± 0.11	5.22 ± 0.13	1.23 ± 0.19	4.31 ± 0.19	4.86 ± 0.30	0.55 ± 0.36

Lower CP intakes (10.4 and 12.4 g CP/bird/day) resulted in weight loss over the 29 weeks of the experimental period in both natural mating and individual pens. Naturally- mated birds receiving 10.4 and 12.4g CP/day were 24.9 and 23.6% below target BW at 59 weeks respectively. Birds in individual cages receiving the same CP intakes were 26.5 and 22.4% below target BW at 59 weeks respectively. Higher CP intakes (17.8- 20.1 g CP/bird/day) however resulted in BW gain over the experimental period. The highest weight gains recorded in both natural- mating and AI scenarios resulted from 20.1 g CP intake. Birds receiving 20.1 g CP/day were 100% on breed target weight in natural mating pens and 13% over breed target weight in individually caged birds. This could be due to the lack of courtship behaviour (looking for females, competition with other males), courting and mating, thus BWs are above recommended target BWs. However, this did not prove to affect fertility and fertility parameters in this experiment.

Although there was no significant response to protein intake for BW at 31 weeks of age in individual cages, there was a significant polynomial response at 45 and 59 weeks of age ($P<0.001$, $R^2= 93.3$) (Figure 1).

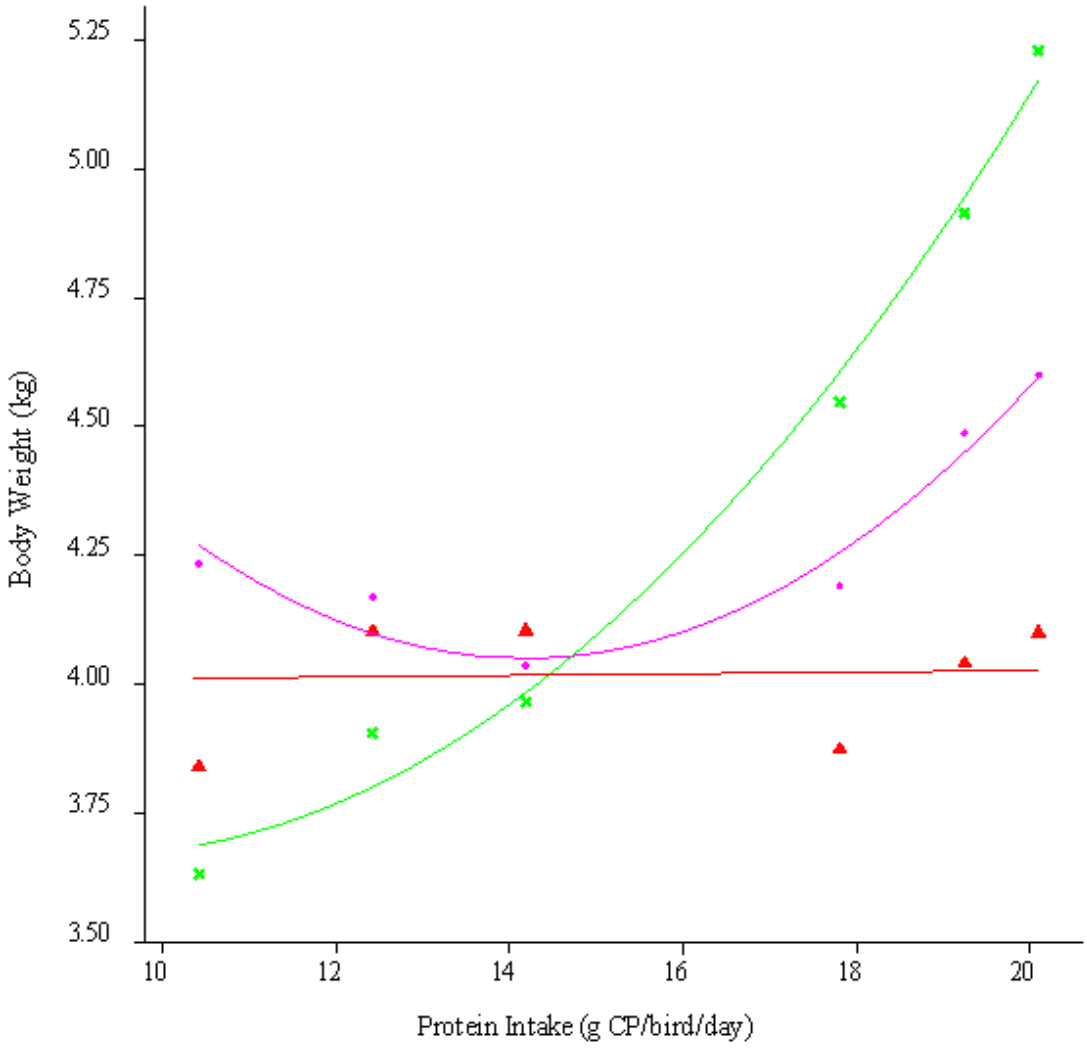

Figure 1. *The mean body weight (kg) of birds in individual cages used for AI, in response to dietary protein intake (g CP/bird/day) during the production cycle at 31 (▲), 45 (•) and 59 (×) weeks*

At 31 weeks of age, BW was not influenced by CP intake, with BW being similar at various CP intakes. This could be the result of the birds not consuming the diet for long enough for effects to be noted. It is clear that BW is influenced to a larger degree by CP intake as the birds' remain on the diet for longer periods and thus BW is significantly influenced by CP intake at 59 weeks of age, and to a lesser degree at 45 weeks of age (Figure 1).

A significant linear response was when comparing mean BWs of birds on different CP diets over a period of 29 weeks in natural mating pens($P<0.001$, $R^2= 65$). There was no response in BW of birds in natural mating pens to CP intake at 31 and 45 weeks (P=0.946 and P=0.94 respectively). However, CP intake significantly affected BW at 59 weeks of age ($P<0.05$), with BW increasing by 2.2 g per g CP intake per day (Figure 2). Curvilinear regression did not improve the goodness of fit for this model, in spite of it being significant.

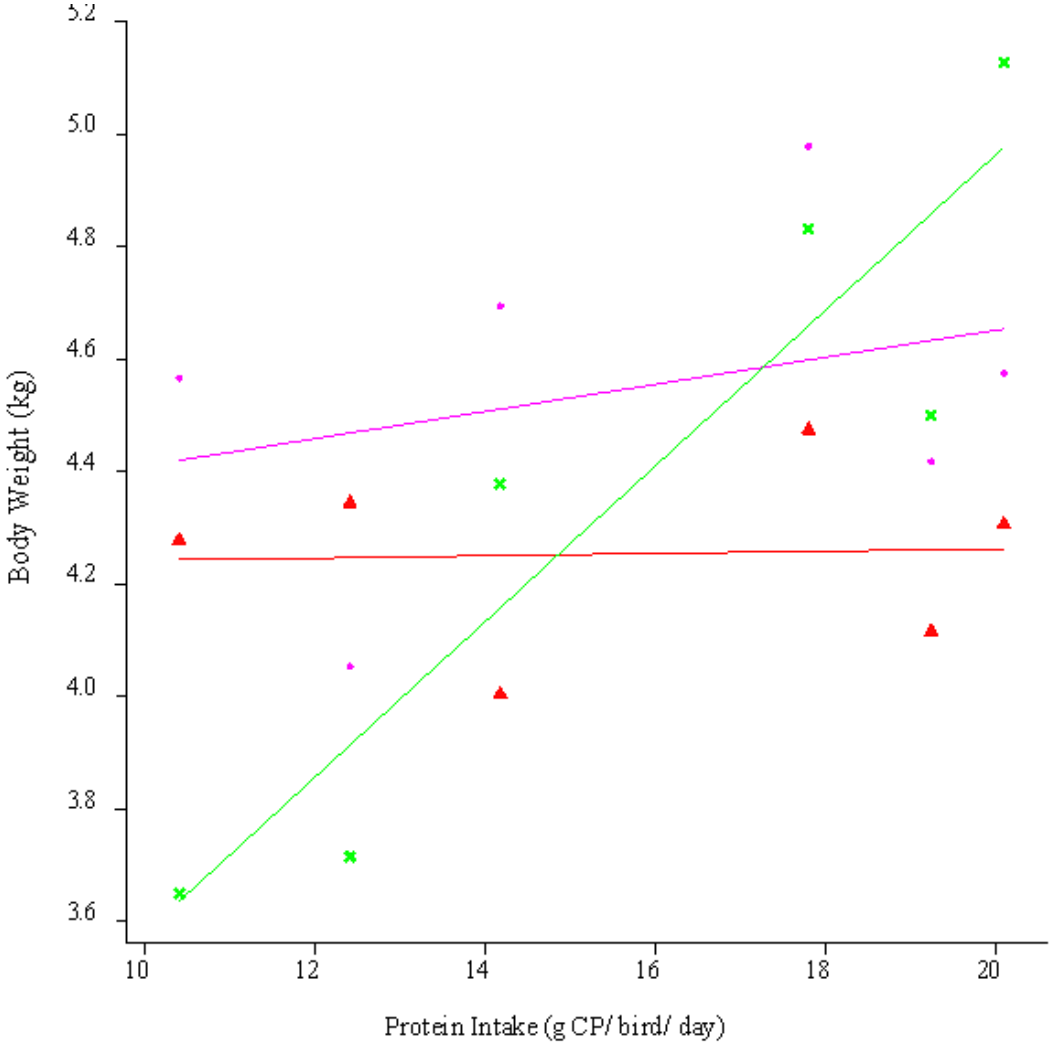

Figure 2. *The body weight (kg) of birds in natural mating pens, in response to dietary protein intake (g CP/bird/day) during the production cycle; at 31 (▲), 45 (•) and 59 (✖) weeks*

The lack of response in BW at 31 weeks could be explained by the short time these birds were fed the experimental diets. The lack of response at 45 weeks could not be explained. It was expected that birds at this age would have started to show a response to the different treatments, if to a lesser extent to that observed at 59 weeks. By the end of production BW was greatly affected by, both a lower and a higher nutritional plane. When birds were fed the lower CP diets, BW was negatively affected, whilst on the higher intakes, BWs were higher which could be due to the excess nutrients being deposited as fat (even though body composition was not measured).

3.4.2 Hatchability of Artificially Inseminated Birds

There was no significant response in hatchability to CP intake in eggs after AI from birds in individual cages. The lack of response could have been due to the low number of eggs set (Table 6).

Table 6. *The hatchability (%) of eggs from artificially inseminated and naturally mated birds; where (n) is the number of eggs set.*

Protein intake (g CP/bird/day)	Hatchability (%)									
	Artificial Insemination				Natural- mating					
	45 weeks	(n)	59 weeks	(n)	31 weeks	(n)	45 weeks	(n)	59 weeks	(n)
10.4	83.3	6	66.7	12	86.0	150	90.0	120	80.0	105
12.4	80.0	5	75.0	12	88.0	150	84.2	120	81.0	105
14.2	83.3	6	100	12	92.7	150	85.0	120	86.0	114
17.8	87.5	8	83.3	12	95.3	150	89.2	120	78.1	105
19.3	75.0	8	75.0	12	82.7	150	86.7	120	90.3	113
20.1	88.9	9	100	12	91.3	150	87.5	120	91.2	114

Results from break-out of hatch debris of artificially inseminated and natural-mating eggs are presented in Table 7.

Table 7. *The break-out of hatch debris of artificially inseminated and natural- mating eggs after the incubation period*

Protein intake (g CP/bird/day)	Hatch Debris (%)									
	Artificial Insemination									
	45 weeks					59 weeks				
	E.E.D[2]	M.E.D[3]	L.E.D[4]	Infertile	(n)	E.E.D[2]	M.E.D[3]	L.E.D[4]	Infertile	(n)
10.4	-	-	16.7	-	6	8.33	-	8.33	16.7	12
12.4	-	-	-	20.0	5	8.33	8.33	-	8.33	12
14.2	16.7	-	-	-	6	-	-	-	-	12
17.8	-	-	-	12.5	8	-	8.33	8.33	-	12
19.3	-	12.5	-	12.5	8	-	8.33	-	16.7	12
20.1	-	-	-	11.1	9	-	-	-	-	12

Protein intake (g CP/bird/day)	Natural- mating														
	31 weeks					45 weeks					59 weeks				
	E.E.D[2]	M.E.D[3]	L.E.D[4]	Infertile	(n)	E.E.D[2]	M.E.D[3]	L.E.D[4]	Infertile	(n)	E.E.D[2]	M.E.D[3]	L.E.D[4]	Infertile	(n)
10.4	3.33	2.67	0.67	6.67	150	2.50	3.33	1.67	1.67	120	2.86	0.95	2.86	13.33	105
12.4	1.33	-	1.33	6.67	150	2.50	2.5	1.67	9.17	120	3.81	2.86	2.86	9.52	105
14.2	3.33	1.33	0.67	2.00	150	3.33	1.67	1.67	6.67	120	1.75	3.51	0.88	7.89	114
17.8	0.67	1.33	0.67	2.00	150	1.67	1.67	1.67	5.83	120	2.86	1.90	2.86	14.3	105
19.3	4.00	-	3.33	10.0	150	0.83	1.67	3.33	6.67	120	1.77	0.88	1.77	5.31	113
20.1	2.00	-	2.67	4.00	150	2.50	1.67	3.33	5.00	120	0.88	0.88	-	7.02	114

2 Early Embryo Death, 3 Mid Embryo Death, 4 Late Embryo Death

3.4.3 Hatchability of Naturally-Mated Birds

There was no significant response to CP intake for hatchability in naturally mated birds. Although the number of eggs set for natural mating birds were sufficient, there were no replications and thus no response was noted (Table 6).

Hatch percentage across all CP treatments throughout the duration of the study was found to be high, ranging between 75- 100 and 80-95 % in AI and naturally mated birds respectively (Table 6), which are all within specified breed target (Cobb- Vantress, 2008a). This coincides with findings from Arscott & Parker (1963), where no significant differences between treatments were found for hatchability of fertile eggs. They also found a high hatch percentage within a range of CP intakes; 16.9, 10.7 and 6.9 % CP (15.72, 9.20 and 6.07 g CP/bird/day respectively) which correlated to 85.9, 85.6 and 83.4 % average hatch percent respectively.

3.4.4 Assessment for $OPVL_{sperm}/mm^2$ in Natural-Mated Pens

There was an overall significant response in mean Log $OPVL_{sperm}/mm^2$ to CP intake for each age ($P<0.05$; $R^2=52.2$), with a significant interaction between CP intake and age. Due to the low values observed from birds on low CP intakes at 31 weeks, which are difficult to attribute to the treatments since the birds were on the experimental feeds for only two weeks, one could speculate that the interaction observed for these measurements would have disappeared if the data showed less variation (Table 8) and hence the significant main effects of CP intake and age were still considered.

At 45 weeks, there was a trend for the increase of 1 $OPVL_{sperm}/mm^2$ per g CP intake ($P=0.09$). At 59 weeks, although the increase in the Log sperm/mm^2 with the increase in CP intake seems less steep (Figure 3), the mathematical rounding also yielded an increase of 1 $OPVL_{sperm}/mm^2$ per g CP intake ($P<0.05$).

Table 8. *Mean $OPVL_{sperm}/mm^2$ values at different protein intakes (g CP/bird/day) in eggs from natural mating pens.*

Protein intake (g CP/bird/day)	$OPVL_{sperm}/mm^2$								
	31 weeks			45 weeks			59 weeks		
	Mean	Minimum	Maximum	Mean	Minimum	Maximum	Mean	Minimum	Maximum
10.4	3.75	0	50.1	2.39	0	5.63	3.99	0	19.1
12.4	1.59	0	7.32	2.54	0	5.53	5.21	0	26.7
14.8	1.84	0	8.16	5.41	0	20.8	6.64	0	25.3
17.8	3.48	0	11.3	4.84	0	11.8	4.76	0	27.9
19.2	4.52	0	23.9	3.36	0	15.2	5.14	0	25.6
20.1	7.52	0	33.2	3.27	0	12.4	5.28	0	18.3

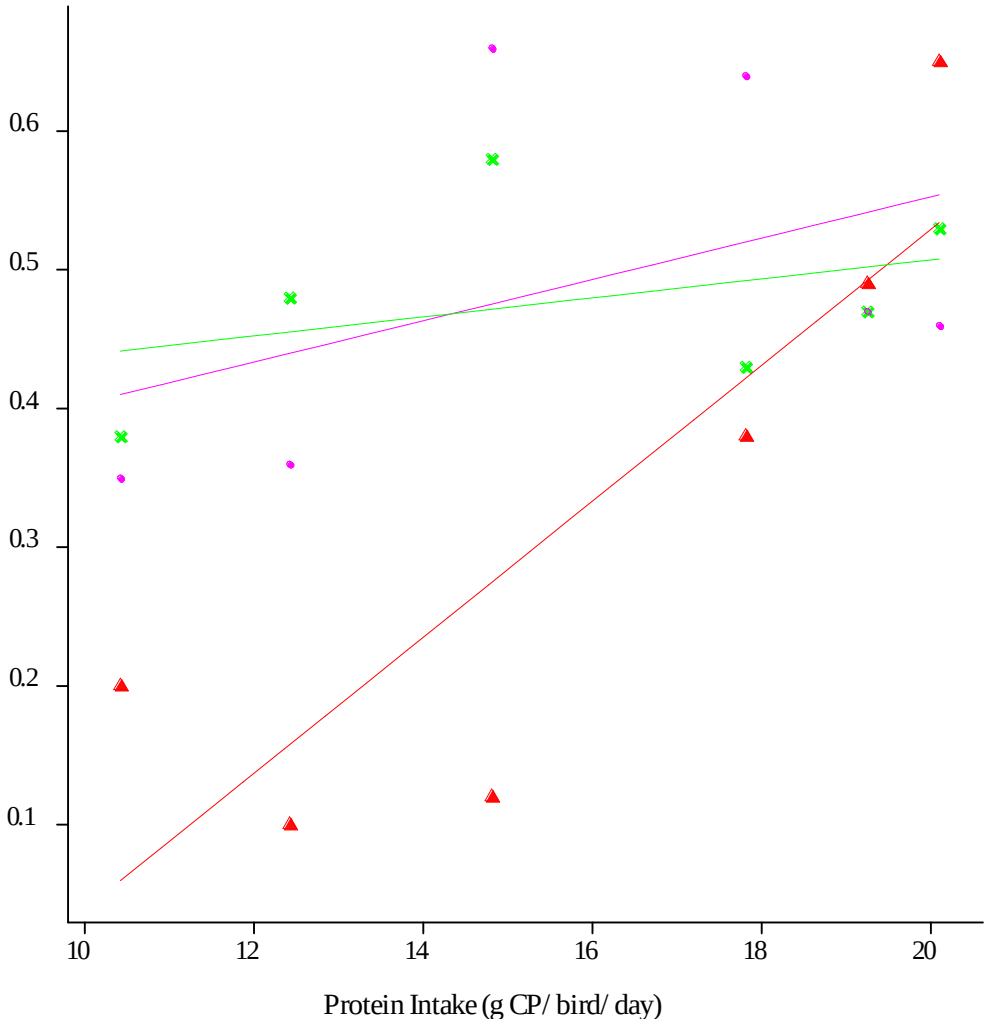

Figure 3. *The mean log OPVL$_{sperm}$/mm^2 values in response to dietary protein intake (g CP/bird/day) at 31 (▲), 45 (•) and 59 (✖) weeks of age*

This method of determining fertility is better to determine a response, as it is continuous data rather than the comparison of binomial fertile versus infertile data received from hatchability.

3.4.5 Egg Fertility Predicted from $OPVL_{sperm}/mm^2$ in Natural- Mating Pens

3.4.5.1 The Response in Fertility to Protein Intake

There was a large variation in data at 31 weeks using the method published by Brillard & Antoine (1990) (Table 9), which probably resulted in the significant interaction found ($P<0.05$) between CP intake and age for this variable. Therefore the significant effects of protein intake at different ages (Figure 4a) were still discussed. Predicted fertility at 45 weeks was higher across all CP intakes, with an increase in predicted fertility of 0.56% per g CP intake ($P<0.001$). Fertility at 59 weeks was overall lower than at 45 weeks ($P<0.01$), with only a 0.18% increase in fertility per g CP intake ($P<0.001$).

Table 9. *Predicted fertility of eggs using methods published by Brillard & Antoine (1990) and Wishart (1997) as well as the breed target for fertility after candling (Cobb-Vantress, 2008b).*

Protein intake (g CP/bird/day)	Predicted Fertility (%)											
	Brillard & Antoine (1990)						Wishart (1997)					
	31 weeks	(n)	45 weeks	(n)	59 weeks	(n)	31 weeks	(n)	45 weeks	(n)	59 weeks	(n)
Breed target	*96.5*	-	*96.1*	-	*91.5*	-	*96.5*	-	*96.1*	-	*91.5*	-
10.4	56.7	30	86.7	30	73.3	30	30.0	30	40.0	30	43.3	30
12.4	53.3	30	83.3	30	80.0	30	23.3	30	43.3	30	50.0	30
14.2	46.7	30	96.7	30	86.7	30	30.0	30	70.0	30	56.7	30
17.8	70	30	96.7	30	73.3	30	50.0	30	76.7	30	50.0	30
19.3	80	30	90.0	30	76.7	30	60.0	30	50.0	30	43.3	30
20.1	90	30	90.0	30	83.3	30	73.3	30	43.3	30	53.3	30

Results of predicted fertility using the method published by Wishart (1997) were similar to findings from the method published by Brillard & Antoine (1990) (Figure 4b). The variation at 31 weeks elicited a significant interaction between CP intake and age ($P<0.05$). There was a tendency for fertility at 45 weeks to increase by 0.92% per g CP intake ($P<0.05$) and a significant response at 59 weeks, with fertility increasing by 0.2% per g CP intake ($P< 0.05$). The shift in

response observed between both methods used (Figure 4a, b), shows the leniency of the method published by Brillard & Antoine (1990) which considers an egg to be fertile with a lower OVPLsperm/mm^2.

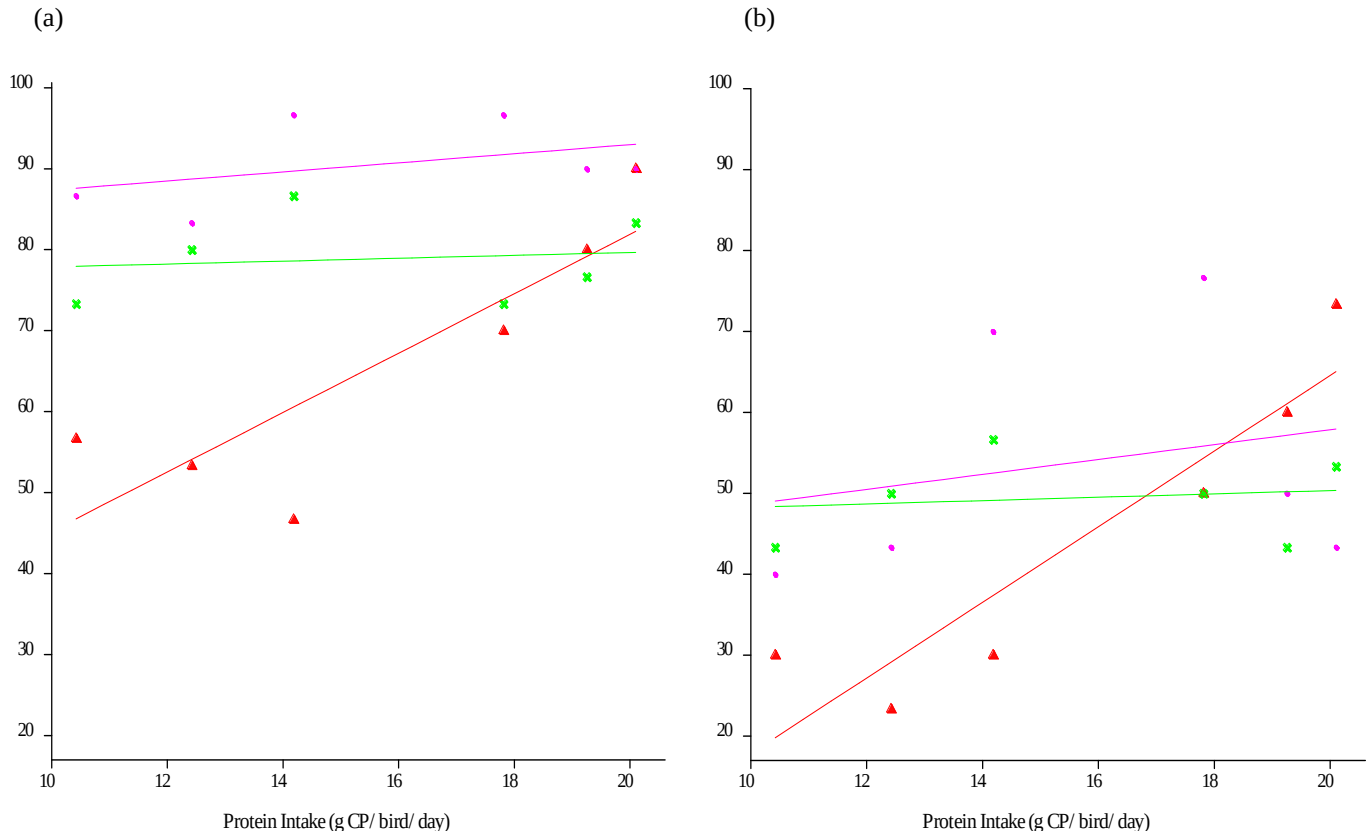

Figure 4a & b. *Fertility predicted using the method proposed by Brillard & Antoine (1990)**(a)** and Wishart (1997)**(b)** through examination of the OPVL$_{sperm}$/mm^2, in response to protein intake (g CP/bird/day) at 31 ( ), 45 ( ) and 59 ( ) weeks of age*

3.4.5.2 The Response in Fertility to Age

No response was found in predicted fertility with age using both methods published by Brillard & Antoine (1990) and Wishart (1997) (Figure 5).

Original analysis included data from week 31, however this yielded an overall improvement in fertility as the birds aged, which was not expected or compatible with the expected fertility curve

for the specified breed (Cobb-Vantress, 2008b). This regression analysis did not result in any significant relationship between predicted fertility and age for each of the CP intake groups. There was large variation between treatment means at 31 weeks which skewed the data, thus these values were excluded from the analyses, as the birds had only been on experimental diets for 2 weeks and it is likely that the variation observed was not due to these diets. Once this data was excluded the response was more meaningful (Figure 5).

Predicted fertility using the method published by Brillard & Antoine (1990), did not result in a significant response, but a trend was found ($P=0.073$), which showed that predicted fertility declined as the birds aged ($P<0.01$) and there was a tendency indicating for birds with a CP intake of 14.2 g CP/bird/day to have the best fertility overall ($P=0.063$). There were no differences in the response for all other CP intake groups.

Similar results were found from the regression analysis for the data using the method published by Wishart (1997) (Figure 5a, b). Figure 5 shows the difference between these two methods of prediction of fertility. The method proposed by Brillard & Antoine (1990) is more lenient in accepting an egg as fertile with fewer OPVLsperm/mm^2, whilst Wishart's method requires more OPVLsperm/mm^2 for an egg to be fertile. This is clear in Figure 5b where the data for lower CP intakes have a lower fertility value at 45 weeks.

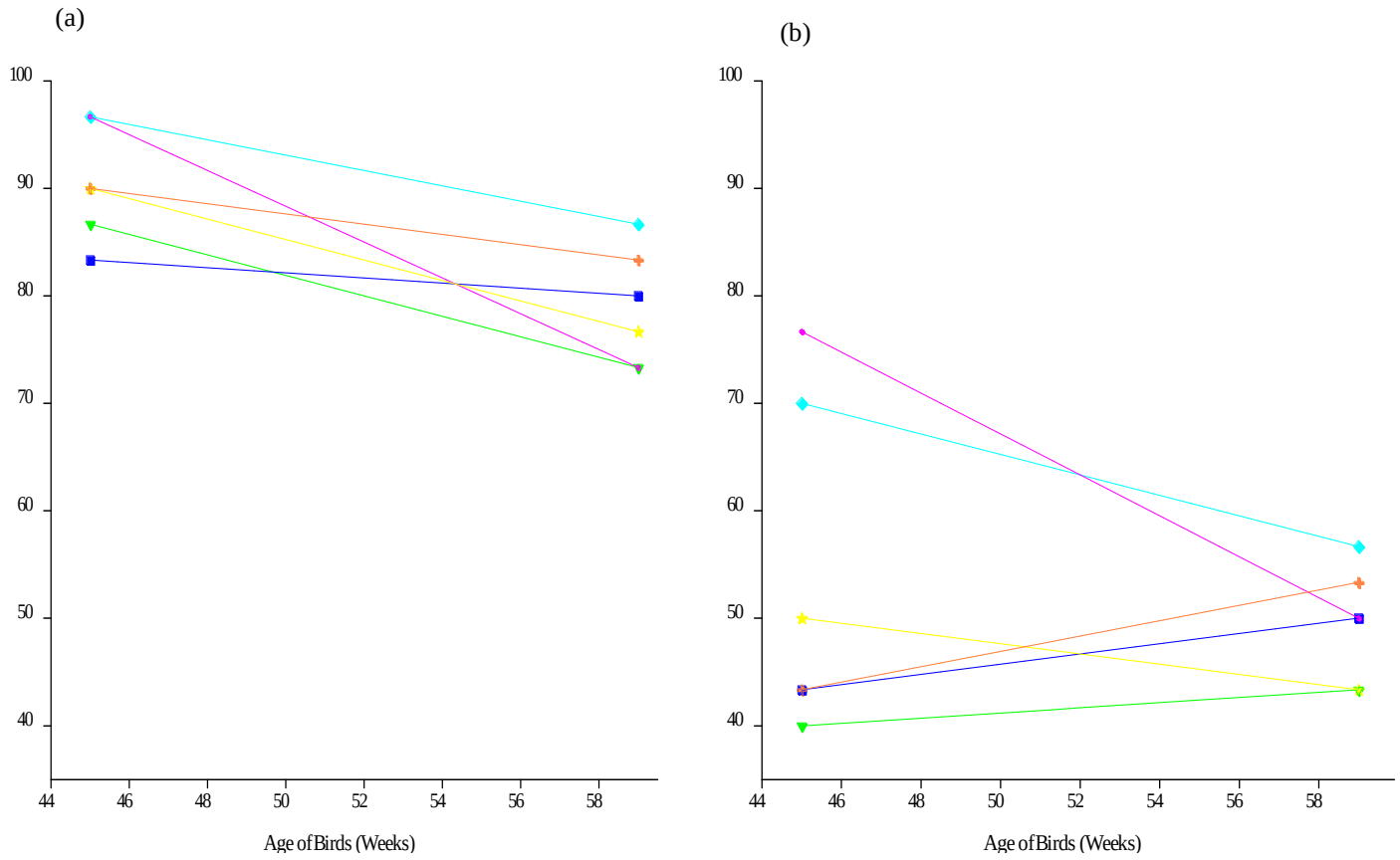

Figure 5a & b. *Fertility predicted using the method proposed by Brillard & Antoine (1990)**(a)** and Wishart (1997)**(b)** through examination of the $OPVL_{sperm}/mm^2$ from 45 to 59 weeks of age, where 10.4 ( ), 12.4 ( ), 14.2 ( ), 17.8 ( ), 19.3 ( ), 20.1 ( ) g CP/bird/day.*

Improved fertility predictions could have been made by using examination of IVPL. Wishart et *al.* (2001) suggested that even though spermatozoa in the vicinity of the germinal disc are visible it does not mean they have penetrated the IPVL.

3.5 Conclusion

Many studies have been conducted over the years to determine the ideal protein intake in a BB male ration which would maximise fertility and reduce feeding cost and nitrogen excretion to the environment. Dietary protein and AA's are some of the most expensive nutrients per unit weight (Coon, 2004; Leeson, 2010). It is therefore crucial to obtain the optimum protein content in a ration. It is however, difficult to determine the optimum CP intake when the birds' requirements are based on older, more out-dated information, considering the genetic potential of chickens has improved significantly (Leeson, 2010).

To maintain optimal breeding performance within the flock, BWs need to be maintained within target recommendations throughout the production cycle, but this becomes more difficult as the birds age with detrimental effects on overall fertility. Results from this experiment show that BW is influenced to a larger degree by CP intake at 45 and 59 weeks of age in both AI and naturally- mated males. It is therefore essential to manage breeder flocks' dietary needs with caution as the birds' age. The impact of the dietary treatments imposed at these ages on BW is apparent from overall variation in BW at lower or higher CP intakes. Birds with the lower CP intake lost weight which may impact flock morbidity and welfare, whereas birds with a higher CP intake gained weight and thus mating activity and libido may be compromised. These findings are consistent with Zhang *et al.* (1999); Mench (2002); Romero-Sanchez *et al.* (2007) and Cobb-Vantress (2008a).

Hatchability was not influenced by the CP intakes studied and therefore it appears that the males' ability to fertilise the hens, whether through AI or natural mating, was not compromised at lower or higher CP intakes. This finding is concurrent with those of Koohpar *et al.* (2010). The performance observed using AI or natural mating was similar, the results obtained in trials where males in individual cages are used could be a valid indicator of what would occur in a flock situation; however the lack of social interaction and mating behaviour remains unaccounted for (however, this comparison is beyond the scope of this study).

The response to CP intake was apparent in $OPVL_{sperm}/mm^2$ for each age. Lower values observed from birds on low CP intakes at 31 weeks were attributed to the short duration on experimental feed (two weeks). At 45 and 59 weeks, there was a trend for the increase of 1 $OPVL_{sperm}/mm^2$ per g CP intake. Similar results for predicted fertility using the methods published by Brillard & Antoine (1990) and Wishart (1997) were found; however Brillard & Antoine (1990) is more lenient requiring less OPVLsperm/mm^2 to pronounce an egg fertile. Although no significant response was found to predicted fertility using both methods as CP intake increased, there was a tendency for predicted fertility to be higher in birds with an intake of 14.2 g CP/bird/day.

Although there was no effect of CP intake on hatchability, there was a tendency for a superior response in predicted fertility from birds with a CP intake of 14.2 g CP/bird/day. This intake also least affected the BW of the male birds at 59 weeks and thus 14.2 g CP/bird/day can be recommended for optimal BB male performance.

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APPENDIX A

Composition of the Tyrodes solution.

Component	Quantity (g)
$Ca\ Cl_2$	0.2 **
$MgCl_26H_2O$	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 1000ml

** add last while stirring to prevent precipitation

The solution must be filtered into a sterile container and stored in a refrigerator. The solution must be discarded after 2 weeks.

APPENDIX B

Composition of Eosin and Nigrosin solution.

- ★ 8 ml distilled water
- ★ 2 drops Eosin *
- ★ 5 drops Nigrosin **

* Eosin- 5 g per 100 ml distilled water

** Nigrosin- 10 g per 100 ml distilled water

APPENDIX C

Formula used to convert the number of sperm in 20 fields of view into mm^2

Sperm per mm^2= A/ B x 1 000 000*

Where:

A- total number of sperm counted in 20 fields of view (e.g. 30 sperm)

B- Total area viewed in 20 fields of view (e.g. 3553743.80 μm^2)

* Conversion factor to convert from μm^2 to mm^2

Example

30/ 3553743.80 x 1 000 000= 8.4 sperm per mm^2