THE EFFECT OF PHOTOPERIOD AFTER PHOTOSTIMUALTION ON MALE BROILER BREEDER FERILITY

by

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As the candidate's Supervisor I agree/do not agree to the submission of this dissertation.

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ABSTRACT

Fertility in male broiler breeders may be controlled through a number of different methods, including lighting programmes. Currently in the poultry industry, male and female broiler breeders are reared under the same lighting conditions, as it is assumed that the males respond to light in a similar manner to females. However, the response to degree of photostimulation has not been as fully investigated in males as in females. The effects of different degrees of photostimulation, applied at 20 weeks of age, on the age at sexual maturity and the semen characteristics of male broiler breeders at the time of female sexual maturity, testosterone concentrations, secondary sexual characteristics and sperm-egg interactions were investigated. Results showed that males did not respond to light to the same extent as that seen in female boiler breeders, as ASM was not significantly affected by the degree of photostimulation applied as the males had probably spontaneously started spermatogenesis before photostimulation. This suggests that males could be photostimulated at a younger age than their female counterparts. A large amount of variation in individual male responses to degree of photostimulation was seen and this was possibly due to genetic variation as a result of selection pressure for meat and not fertility traits. Comb area was affected by the testosterone concentration and age but was found to be unrelated to semen characteristics. Semen characteristics were generally not affected by the final photoperiod apart from the semen concentration measured at female ASM. At 51 weeks of age, testes weights were affected by the degree of photostimulation with a trend of lower testes weights from birds on longer photoperiods which suggests the earlier onset of adult photorefractoriness in birds on longer photoperiods. Both testes and comb regression was evident by 51 weeks from at least one bird on each treatment which is concerning for the breeder industry where birds are usually kept for 60 weeks. The numbers of sperm trapped in the perivitelline layer of a hen's egg, and the probability of the egg being fertile, were significantly affected by the photoperiod, age of the male and the time elapsed between artificial insemination and the laying of the egg. The impact of the degree of photostimulation on male broiler breeder fertility is an important topic for the poultry industry and is discussed.

PREFACE

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 2007 to December 2008, under the supervision of Mrs Nicola Tyler.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

DECLARATION - PLAGIARISM

I, Megan Helen McPhail declare that

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

CHAPTER 1

General Introduction

1.1 INTRODUCTION

In general, broiler breeder selection criteria have mainly been based on production traits such as rapid growth and feed conversion to improve the efficiency of the broiler industry. However, due to the negative correlation that exists between growth and reproductive traits, broiler breeder flock fertility is not particularly high. Considering that it takes a substantial amount of time to pass these selected genetic traits down the line from great-grandparent stock to parent broiler breeder stock, and that the expression of the genes in the phenotype is affected by the environment in which the bird is in, it seems that, in the interim, looking at alternative strategies to improve fertility are necessary, as well as allowing improvements in productive traits.

Broiler breeder fertility may be affected positively or negatively, by a number of different environmental variables such as disease, stocking density, feeding regime and lighting procedures (Edens, 1983; Aviagen, 2001; Ciacciariello, 2003; Tolkamp *et al.*, 2005). Disease is relatively easy to control through good managerial practices and the use of vaccination programmes and biosecurity. Stocking density and feeding regimes are basically managed by following the guidelines distributed by the primary breeding companies. Lighting schedules are also provided for broiler breeders by the breeding companies but these lighting profiles have been designed based on experimentation in females and egg laying with breeds such as turkeys and laying hens being considered and not broiler breeders. Some of the suggested lighting programmes are contradictory to the recently published literature and there are areas of discrepancy such as a 15 or 16 h photoperiod at 23 to 25 weeks of age (Lewis, 2006) which is past the saturation daylength and may accelerate the onset of adult photorefractoriness (Lewis and Gous, 2006). Flock fertility is dependent on the fertility of both female and male birds. To date, very little information is available concerning the effects of lighting on male fertility.

When assessing the fertility of males, general practice is to evaluate the size of the testes in order to predict the capacity of the testes to produce sperm. However, in male birds, the

testes are internal and are immeasurable unless the bird is slaughtered. To circumnavigate this problem, theories have been put forward and tested as to whether secondary sexual characteristics, such as comb and wattle area or spur length, could be used to predict the fertility of the male. Other ways to assess male fertility are through semen analysis or sperm-egg interactions. Besides automated semen assessment equipment, which is very expensive, manual semen analysis is subjective and often unrepeatable if different assessors are used.

Thus this trial was designed to primarily examine the effects of lighting on male broiler breeder fertility and to determine if different degrees of photostimulation affected the age at sexual maturity (ASM) and subsequent fertility of the males. Combined with this, secondary sexual characteristics of comb area and the number of primary feathers on the wings were assessed to determine if these characteristics could be used to predict the fertility of the male with the possibility of them becoming selection trait indicators. Both semen analysis and sperm-egg interactions were assessed to determine the fertility of the male broiler breeders.

The literature pertaining to male broiler breeder fertility and the effects of lighting thereon are reviewed and included in Chapter 2. In Chapter 3 the details and findings of this trial can be found. A general discussion where the findings of the trial along with the relevant literature are discussed and can be found in Chapter 4.

CHAPTER 2

LITERATURE REVIEW

2.1   INTRODUCTION

As parent stock, broiler breeders are selected to produce the maximum number of vigorous day-old chicks by combining good fertility, high egg numbers and good hatchability (Aviagen, 2001). Flock fertility is affected by both the male and female birds within the flock as well as external factors such as environmental conditions and disease. This review will focus on the effects of lighting on male broiler breeders.

For fertility to be at a maximum in the male broiler breeder, both the male reproductive and endocrine systems need to function at their maximum potential. Failure of either system to achieve this may lead to a decrease in fertility of the male or even cause the male to become infertile. To date, many different tests have been developed which allow for the evaluation of male broiler breeder fertility. The tests range from simple analysis of semen, such as, colour, volume, motility and morphology, to complex analysis systems which involve the use of computers to track sperm paths or sperm quality. However, the only definite test for fertility involves the production of offspring. Based on this principle, tests which involve the interaction between sperm and the hen's egg have been developed. Some researchers have suggested that secondary sexual characteristics have the potential to predict fertility of a male rooster. However, the literature is controversial and will be discussed in this review.

Male fertility can be affected by a number of different factors, however, in broiler breeders, the main factors are restricted feeding and lighting. Restricted feeding allows for the bird to grow according to a bodyweight-by-age growth curve which is recommended by the primary breeding company. Restricted feeding is a very important topic on its own, and the extent of restriction can alter the age at sexual maturity (Ciacciariello, 2003) but it is only discussed in minimal detail in this thesis. Photorefractoriness is a condition which needs to be considered when a lighting programme is being designed for broiler breeders. A large

amount of literature is available concerning the effects of lighting on female broiler breeder production; however there is less information concerning the male broiler breeder.

The objective of this chapter is to review the relevant research which has been conducted to date on the factors affecting male broiler breeder fertility, with special reference to lighting, and the methods which are available to analyse this fertility.

2.2 MEASURING THE FERTILITY OF THE MALE BROILER BREEDER

The main objective of the broiler breeder industry is to produce the maximum number of viable chicks. In order to achieve this goal, the fertility of the male and female broiler breeder needs to be at its highest and remain high throughout lay. The problem which the industry faces in regards to achieving this goal is that of determining which males have a high fertility, which birds to use in the breeding flock and which birds to cull. The reproductive capacity of a male requires the correct functioning of the reproductive and endocrine systems. Failure of either system to function correctly could lead to a decline in fertility or worse, the complete cessation of function. Many techniques have been developed which allow for the assessment of male bird fertility and will be reviewed. Semen analysis forms the backbone of assessing fertility, where volume, colour, concentration, morphology, motility and mobility are considered. More advanced techniques have been developed which allow for more than one aspect of semen analysis to be considered at a time. Some of these methods include computer assisted sperm analysis and sperm-egg binding assays. Alternative methods for evaluating fertility have also received attention and will be discussed. These include the role of hormones in fertility and the use of secondary sexual characteristics as indicators of fertility.

2.2.1 Male anatomy

The main purpose of the male reproductive tract is to manufacture sperm of a high quality in order to produce viable offspring after transfer to and fertilisation in the female tract. The male bird has an internal reproductive tract, which consists of two bean-shaped testes located on either side of the vertebral column in the abdominal cavity (Kumaran & Turner, 1949a) between the bottom of the lungs and the top of the kidneys (Rose, 1997). Associated with each testis are the epididymis and the ductus deferens, which end in the copulatory organ, situated in the cloaca (Rose, 1997). The greater part of the testis is

4

occupied by seminiferous tubules, where spermatogenesis occurs, and the interstitial cells of Leydig, which are responsible for the secretion of testosterone (Kumaran & Turner, 1949a; Rose, 1997). Sperm and seminal fluid pass from the testes into the ductus deferens. The ductus deferens have four major functions which include the transport of the semen from the testes to the cloaca, the maturation of sperm, storage of sperm prior to ejaculation and the re-absorption of semen which has been stored for too long and thus has been broken down (Rose, 1997). The ductus deferens leads to the cloaca. The copulatory organ is normally concealed within the transverse folds of, and located on the ventral wall of, the cloaca, which can be described as a ball of white gristly tissue (Burrows & Quinn, 1937; Rose, 1997). On either side of the ventral midline of the copulatory organ, erectable tissue can be found, which when erect, results in the ventral midline forming a furrow through which the semen may flow (Burrows & Quinn, 1937). The erection of the tissue causes the folds in the cloaca to straighten out which in turn causes the copulatory organ to protrude from the cloaca (Burrows & Quinn, 1937).

2.2.2 Sperm production

Male birds have two internal testes and thus produce viable semen at a core body temperature of 43ºC (Rose, 1997). De Reviers and Williams (1984) have summarized the trends in testicular development and the onset of spermatogenesis into three phases which include the prepubertal phase, the pubertal phase and sexual maturity. The prepubertal phase (up to 10-14 weeks of age) consists of the slow growth of the testis, confirmed by Parker *et al.* (1942), in the White Leghorn and New Hampshire cockerels and by Aire (1973), in Nigerian and White Leghorn cockerels, which is correlated with age and the increase in body weight, and the proliferation of the undifferentiated Sertoli cells. The pubertal phase (10-14 to 20-24 weeks of age) is characterised by the sharp increase in the rate of testis growth (Parker *et al.*, 1942), a higher spermatogenic activity and the production of the first sperm. Male sexual maturity is reached when testis growth is completed and sperm quality and number are high (Parker *et al.*, 1942).

Sperm production is affected by a number of factors which include the frequency of collection, the age of the bird and the size of the testes (Burrows & Titus, 1939). With more frequent collections, the total semen yield is increased per male and a more efficient reproductive capacity is reached when a higher rather than a lower semen collection

5

frequency is employed (Burrows & Titus, 1939; Noirault & Brillard, 1999). Semen production tends to rise, peak and decline steadily with an advance in the birds age until total sterility is reached (Pearl, 1917). Burrows and Titus (1939) found a high correlation (r^2= 0.78) between testis weight and semen production as did Wilson *et al.* (1988) (r^2= 0.79). However, Onuora (1987) found that the left and right testes have different spermatogenic potential, with the left testis producing sperm earlier and the right testis having more spermatogenic activity when total sperm per mg testis were compared.

Sperm production may be categorised as either being the total number of sperm collected over a period of time and expressed on a "per day" basis, the daily sperm output (DSO), or the total number of sperm produced per day by the two testes, the daily sperm production (DSP) (de Reviers & Williams, 1984; Amann, 1999). The DSP depends only on physiological factors while the DSO is largely dependent on technical factors, such as the skill of the person collecting the semen sample and has nothing to do with the birds ability to produce sperm (de Reviers & Williams, 1984). Determination of the DSP requires knowledge of the lifespan of the spermatids in the seminiferous epithelium as measured by autoradiography, compared to the determination of the DSO which can be performed on collected semen (de Reviers & Williams, 1984). Due to testis size differing greatly among males, DSP also differs greatly (Amann, 1999). Unlike mammals which have external testes that can be easily measured, chickens have to be slaughtered in order to obtain testicular measurements which is not desirable. However, based on 10 ejaculates, the number of sperm per ejaculate divided by two (assuming semen is collected every second day) will give a value for the DSO which can be used as an estimate of the DSP (Amann, 1999). Unfortunately the determination of the DSO is a time-consuming process, which makes it expensive (de Reviers & Williams, 1984), and thus it is not a desirable method for determining sperm production.

2.2.3 Sperm appearance

Poultry sperm can be described as being filamentous in shape, about 100µm in length and as having a small cylindrical head (Rose, 1997). Sperm produced by poultry may be broken down into specific regions, namely the acrosome, the head, the neck, the midpiece containing mitochondria for sperm metabolism and the tail which allows for sperm movement by flagella action (Lake *et al.*, 1968; Bakst & Howarth, 1975). The microscopic

examination of poultry sperm reveals that there are differences between the structure of the fowl sperm and that of mammals with respect to size, shape and ultrastructure (Lake *et al.*, 1968). The junctions of the acrosome with the head, the head with the tail and the midpiece with the principle-piece of the tail are not indicated by noticeable changes in diameter as they are in the sperm of most mammals (Lake *et al.*, 1968).

The acrosome is approximately 2.5µm in length, the length of the head is 12.5µm, the midpiece is 4.3µm and the tail is approximately 90µm long (Lake *et al.*, 1968). The diameter of the head and the midpiece is about 0.5µm at the widest point (Lake *et al.*, 1968). The acrosome is a well defined region of the fowl sperm and consists of an acrosome cap and an acrosome spine (Lake *et al.*, 1968) and can be described as being conical in shape (Bakst & Howarth, 1975; Maeda *et al.*, 1986). The acrosome is composed of a homogenous matrix which is bounded by a singe unit-membrane while the acrosome spine is composed of a relatively dense substance (Lake *et al.*, 1968; Bakst & Howarth, 1975). The head has a curved, filiform shape (Lake *et al.*, 1968) and contains an elongated nucleus which is enveloped in a double membrane (Bakst & Howarth, 1975). In 1968, Lake *at al.* defined the neck of fowl sperm as being short and composed of a complex centriolar structure from which the fibres of the tail began. However, in 1975, Bakst and Howarth redefined the fowl sperm neck as including the space occupied by the centriolar complex and the non-striated connecting-piece. In the midpiece of the sperm approximately 30 mitochondria (Bakst & Howarth, 1975) surround the axial fibres (Lake *et al.*, 1968) and these allow for metabolic reactions to occur. The tail of the sperm has the most important function (Lake *et al.*, 1968) as it becomes motile upon ejaculation which is essential for the sperm to reach the site of fertilisation. Lake *et al.* (1968) defined the tail as having four regions, the neck, midpiece, principal piece and a very short endpiece. The principal piece is thought to influence the type of movement of the sperm (Lake *et al.*, 1968). The ultrastructure of sperm plays a large role in the determination of infertile birds in that a bird which is known to be infertile may appear to have normal sperm upon observation under light microscopy, but by examining the ultrastructure of the sperm, certain ultrastructural defects may be observed.

2.2.4 Semen collection

Unlike domestic mammals which have a well defined penis during ejaculation that allows for the use of a teaser animal or an artificial vagina for semen collection and fractioning, the male fowl possesses a cloaca which makes the collection of semen more difficult (Burrows & Quinn, 1937; Lake, 1957). The fowl copulatory organ is complex in that it consists of ejaculatory ducts, lymph folds, vascular bodies and a phallus (Lake, 1957). Lumbar massage causes the organ to become erect and to protrude from the cloaca after which it is squeezed and the fluid flowing into collecting vessels is considered to be semen (Lake, 1957). The first relatively easy method for the collection of semen from the male fowl was proposed by Burrows and Quinn in 1937. The method is considered to be a two-man technique in which the bird is stimulated to ejaculatory response by massaging the soft sides of the abdomen between the gizzard and the pelvic bones coupled with the massaging of the back and the gripping of the copulatory organ upon protrusion in order to milk out the semen contained in the bulbous ducts. However, Burrows and Quinn (1937) did note that the semen sample may become contaminated with urine or faeces and that the males varied greatly in their response to the method. Twenty years later, in 1957, Lake described a single man method for the collection of fowl semen. This method entailed placing the bird between the knees of the operator, sitting in a low chair or squatting against the wall. The lumbar region of the back is massaged in a downwards fashion using the right hand after which the same hand is moved to the cloaca to protrude the erect copulatory organ in order to collect the semen in a glass vial. In general, the best response from the male is obtained after the bird has had a certain amount of training as this will limit the chances of urination and defecation as well as allow production of a sample which is of a good quality (Lake, 1962). The abdominal massage method of semen collection which was introduced by Burrows and Quinn (1937) forms the basis of the semen collection techniques which are used today. Although modifications of this method have been made, the objective of semen collection remains the same; to collect high quality semen from the male fowl with minimal amounts of contamination (Sexton, 1983).

2.2.5 Evaluation of fertility

The selection of male birds for breeding flocks is usually based on certain physical characteristics which include body conformation and size, eye health and the appearance of the head (Wilson *et al.*, 1979). Even though these characters are useful in selecting a

male bird for breeding, they may not give a true reflection of the fertility or actual reproductive capacity of the bird. Many assessments to determine this include the evaluation of the quality of the semen. Semen evaluation of males allows for a breeder to eliminate poor semen producing males from the breeding flock (Sexton, 1983). Attempts have been made to relate various semen quality characteristics to fertility but there is disagreement in the literature regarding the importance and reliability of semen traits in predicting fertility (Wilson *et al.*, 1979). The fertility or breeding potential of a male is also influenced by genetic, physiological and social factors (Wilson *et al.*, 1979). Seasonal changes in the quality of semen may be experienced (Lake, 1962) and these changes need to be considered when evaluating a semen sample from a male bird. Seasonal differences have been observed in Rhode Island Red males in the volume and density of the semen as well as in the total number of sperm produced with a decrease in sperm production in winter (Parker & McSpadden, 1943). As yet, there is no single laboratory measurement of semen quality which will predict unfailingly the fertilising capacity of a semen sample (Sexton, 1983; Graham, 2001). Although Froman *et al.* (1999) concluded that sperm mobility is a primary determinant of fertility in the fowl, most authors (Lake, 1989; Gill & Amann, 2002) suggest rather that a number of tests should be used together to evaluate a male for breeding as one sperm may fail to fertilise an ovum due to a morphological reason and another sperm may fail for a different reason such as motility (Gill & Amann, 2002). There is a large amount of literature available concerning different methods of semen evaluation, but this review is concerned with the more common methods which are used in practice today. These methods include principal parameters of semen evaluation such as sperm concentration, number of live/ dead sperm and motility as well as advanced methods like sperm-egg binding assays and mobility assays. Ideally semen evaluation tests should be objective, quantitative, simple to perform and interpret and inexpensive (Lake, 1989). However, for poultry production, evaluation from individuals is generally limited to the visualisation of semen colour and measurement of ejaculate volume and sperm concentration (Amann, 1999; Donoghue, 1999) as these are the semen characteristics which are relatively easy to measure (Gill & Amann, 2002). The important sperm attributes (capability to bind to the perivitelline layer of an ovum, percentage excluding dye and mobility or wave action) (Gill & Amann, 2002) require more complicated equipment and laboratory tests which are time consuming if a producer is looking for an immediate answer.

<u>2.2.5.1 Principal parameters of fowl semen evaluation</u>

The principal parameters of fowl semen analysis usually consist of an evaluation of the volume, colour, pH, motility, morphology and percentage of live and dead sperm in the semen sample. A number of different methods have been developed which allow for these assessments. Some of these methods are still used today, however, others have a limited use in that they require laboratory facilities and/or lack the robustness required for use on the farm (Hazary & Wishart, 2001).

2.2.5.1.1 *Volume*

The volume of semen may be measured by collecting the sample in a conical tube and recording the volume using the measurements on the tube (Kirby *et al.*, 1998), with a pipette (McDaniel & Craig, 1959), directly with a graduated collecting tube (Cooper & Rowell, 1958) or indirectly by measuring the difference in weight between an empty and filled sample cup (Bilcik *et al.*, 2005). Sperm loss, experienced in the pipette method, can be minimised by using a small, preweighed disposable collection cup and determining seminal volume gravimetrically (Amann, 1999). The volume of semen produced seems to vary with each individual bird as well as with the age of the bird, in that older birds produce larger amounts of semen than younger males (Romanoff, 1960). Male domestic fowl produce 0.5-1.0ml of semen per ejaculate, according to Rose (1997). Burrows and Quinn (1937) reported that individual output ranged from 0.4c.c. to 3.6c.c. of semen at a single collection with the exceptional individual producing 4.0c.c. at a single collection. However data from Parker and McSpadden (1943) show that the mean volume of semen produced varied from 0.36c.c. to 0.93c.c. compared to Burrows and Titus (1939) who reported a variation from 0.42c.c. to 1.27c.c.

2.2.5.1.2 *pH*

A pH meter can be used to measure the pH of poultry semen (Cooper & Rowell, 1958), which varies between 7.0 and 7.6, depending on the amount of transparent fluid that mixes with the semen during collection (Cooper & Rowell, 1958; Rose, 1997). The measurement of pH can be indicative of infection if abnormal values are recorded, and therefore may be helpful in prescribing an antibiotic (Johnston, 1991).

2.2.5.1.3 *Sperm motility and mobility*

Sperm motility is expressed as the percentage of total motile or progressively motile sperm and it should be evaluated immediately after collection (Freshman, 2002). Originally, motility was assessed subjectively using light microscopy but advances in research have lead to the development of four principal means of evaluating sperm motility: spectrophotometry, videomicroscopy, digital image analysis (Holm & Wishart, 1998) and the movement of sperm from one medium to another (Froman & McLean, 1996). Both Wall and Boone (1973), and Wishart and Ross (1985) described modified versions of Timourian and Watchmaker's (1970) spectrophotometric technique, originally used for measuring the motility of sea urchin sperm, which allows for the estimation of fowl and turkey sperm motility. The spectrophotometric technique depends on the rheotactic and light-scattering properties of sperm when subjected to a beam of light (Wishart & Ross, 1985). Wall and Boone (1973) used their method to determine a motility index which they found to have a high correlation with percent motility and vigour. The method of Wishart and Ross (1985) allows for the calculation of the concentration of sperm, the percentage of motile sperm and the rate or vigour of movement of the motile sperm within a sample. The limited use of videomicroscopy and digital image analysis is most likely linked to the cost of the instrumentation (Froman & McLean, 1996). The movement of sperm from one medium to another forms the basis of the motility assay developed by Froman and McLean (1996). Accudenz is a non-ionic, biologically inert, cell separation medium which undergoes a change in absorbency when it is overlaid with a sperm suspension (Froman & McLean, 1996). When a semen sample is overlaid upon Accudenz, immotile sperm do not enter the layer while motile sperm enter Accudenz rapidly thus resulting in the increase in the absorbancy (Froman & McLean, 1996; Froman *et al.*, 1999) The advantages of using Accudenz are that it approximates physiological conditions, requires portable equipment, it is applicable to individual males and it is highly repeatable (Froman & McLean, 1996). The sperm penetration of Accudenz measures the size of motile subpopulations of sperm within a sample as the sperm overlay contains a fixed number of sperm, the incubation time is constant and the absorbance of the Accudenz layer is directly proportional to the number of sperm that enter it from the sperm overlay (Froman *et al.*, 1997).

Froman and McLean (1996) stated that spectrophotometry was one of four principal methods used to assess the motility of poultry semen. Where other methods of measuring motility have been based upon the rheotactic properties of sperm (Wishart & Ross, 1985), Froman and McLean (1996) used spectrophotometry to quantify the net movement of sperm through a layer of Accudenz at body temperature (Froman & Feltmann, 1998). This allowed for the penetration of sperm into Accudenz to become a means of quantifying sperm mobility (Froman & Feltmann, 1998; Bowling *et al.*, 2003) rather than motility. Sperm mobility is defined as the progressive swimming motion or net movement of a population of sperm through a viscous medium (Birrenkott *et al.*, 1977; Froman *et al.*, 1999; Froman & Feltmann, 2000; Dumpala *et al.*, 2006) and it is a quantitative trait of the domestic fowl (Froman & Feltmann, 1998) as well as being a biologically significant predictor of broiler breeder semen quality (Bowling *et al.*, 2003). The measurement of poultry sperm motility by sperm penetration of Accudenz does not offer information about individual sperm but rather it is an assessment of the mobility of a population of sperm (Froman & McLean, 1996). It is sperm mobility rather than sperm motility that allows for the sperm to reach the hens storage tubules (Froman & McLean, 1996). Froman *et al.* (1999) concluded that sperm mobility is a primary determinant of fertility in the fowl.

2.2.5.1.4 *Sperm morphology*

The quantification of morphologically abnormal sperm has been used as a method of assessing avian sperm quality (Bilgili *et al.*, 1985a). Morphology is described as the percentage of sperm which display a normal appearance. Morphology of the sperm may be analysed in order to determine if there are any abnormalities which may result in a decreased fertility of the bird. By examining a stained semen smear (Bilgili *et al.*, 1985a) under a light microscope and counting 100 to 200 sperm in which the numbers and types of abnormal morphology are recorded and the percentage of normal sperm are calculated, allows for morphology to be assessed (Freshman, 2002). Abnormalities may be described as primary, occurring during spermatogenesis or secondary, occurring during passage through the epididymis or during collection and preparation of the slide (Kirby *et al.*, 1990; Freshman, 2002). Abnormalities may affect different regions of the sperm cell such as bent, broken or curled (Clarke *et al.*, 1984) tails; swollen, hooked or ruptured heads and crooked-necks or malformed midpieces (Bilgili *et al.*, 1985a; Edens *et al.*, 1973). In a

typical sample of fresh semen, abnormal or nonviable sperm vary between 5 and 20% (Bakst *et al.*, 1994).

2.2.5.1.5 *Sperm concentration*

Sperm concentration is a characteristic of semen and can be influenced by the skill of the semen collector, the amount of transparent fluid obtained and by the number of cells in the sperm-rich material (Gill & Amann, 2002). Sperm concentration can be determined by the use of a haemocytometer (McDaniel & Craig, 1959) but this method may be influenced by subjectivity depending on the number of squares counted. Other disadvantages of this method are that a large number of cells need to be counted in order to be accurate and a large dilution of semen has to be made, which introduces variation, unless the sperm are suspended equally in solution (Den Daas, 1992). Most routine measurements of concentration are made with a spectrophotometer in which the degree of light scattering is related to a calibration curve from which concentration can be extrapolated (Den Daas, 1992). A more objective measurement of sperm concentration is fluorometry[1] (Bilgili & Renden, 1984).

2.2.5.1.6 *Differentiation of live from dead sperm*

The laboratory evaluation of semen quality often involves the determination of the percentage of live and dead sperm (Wilson *et al.*, 1969). Stains such as nigrosin-eosin may be used to evaluate the percentage of dead sperm and thus by default the percentage of live sperm. Sperm which take up the dye to any extent are considered to be dead (Cooper & Rowell, 1958). However, sperm are known to die relatively soon in this stain (Wilson *et al.*, 1969) and this brought about the development of a new stain, trypan blue. Wilson *et al.* (1969) describe this stain as being simple, fast and reproducible as well as being valid for the identification of dead sperm within a semen sample.

Cooper and Rowell (1958) presented data to show that fertility is significantly correlated with the percentage of dead sperm and that the quality of semen, as measured by the percentage of dead sperm, determines the fertilising ability to a greater extent than the percentage of live sperm. The authors reasoned that if a male produced semen with a high

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[1] Refer to section 2.2.5.1.7 *The use of dyes in semen evaluation* page 15

proportion of dead sperm, then the likelihood of the live sperm having a poor fertilising capacity was high as they found a high correlation (r = -0.93) between the percentage of dead sperm and the motility of the live sperm, independent of the density of live sperm.

2.2.5.1.7 *The use of dyes in semen evaluation*

The ideal tests for measuring the quality of semen from poultry should be objective, quantitative, simple to perform and interpret and be inexpensive (Chaudhuri & Wishart, 1988). Originally, staining was, and still is, used to assess sperm quality. However, laboratory use of staining techniques is limited by labour and time requirements (Bilgili & Renden, 1984), results in subjectivity, as examiners are easily influenced by stain characteristics (Bilgili *et al.*, 1985a), and accuracy and repeatability are easily influenced (Bilgili & Renden, 1984). This lead to the development of dye-reduction tests. Chaudhuri and Wishart (1988) developed a technique by which the reduction of a colourless tetrazolium dye, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl-tetrazolium chloride (INT), to a red formazan pigment by fowl sperm can be monitored in a controlled manner. This technique is a controlled, quantitative and objective test (measuring the colour change spectrophotometrically rather than by eye) which allows for the metabolic activity of fowl sperm to be measured (Chaudhuri & Wishart, 1988). After further research, Chaudhuri *et al.* (1988) found that the rate of INT reduction by fowl sperm was significantly correlated with sperm motility, ATP content, morphology and fertilising ability (estimated by candling eggs and hence calculating the proportion of fertile eggs laid per hen inseminated). Hazary and Wishart (2001) criticised the method of Chaudhuri and Wishart (1988) by saying that the reagents involved in the test include an unstable phenazine methosulphate (PMS) and a highly toxic potassium cyanide (KCN) and that the samples had to be centrifuged before they were optically clear enough to be read by the colorimeter. They proposed an assay whereby fowl sperm reduce the dye 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT). In this assay the unstable and toxic reagents have been removed and there is no longer a need to centrifuge the sample as a reagent has been added which clears the sample sufficiently for the absorbance to be read (Hazary & Wishart, 2001). The MTT reduction assay was later compared with other assays of sperm quality and fertilising ability and it was found that high correlations existed between MTT reduction potential and ATP content (r=0.85), ability to penetrate Accudenz (r=0.62), hydrolytic activity towards the

14

inner perivitelline layer (r=0.80) and fertilising ability of sperm (r=0.83) (measured by the percentage of fertile eggs after insemination) (Hazary *et al.*, 2001).

Another dye technique which can be used to assess fowl sperm is fluorometry. This technique is based on two principles; the nuclear stain ethidium bromide (EtBr), binds double-stranded, undenatured nucleic acids of sperm as a intercalating dye and causes an intense fluorescence, which is proportional to the concentration of nucleic acids and, secondly, EtBr is excluded from viable cells by the intact cell membrane (Bilgili & Renden, 1984; Gliozzi *et al.*, 2003). The advantages of this technique are that only a small amount of semen is required, it provides an accurate and simultaneous estimation of fowl semen viability and concentration (Bilgili & Renden, 1984), it provides a rapid and objective assessment of sperm integrity and it has a reasonable cost (Gliozzi *et al.*, 2003). After comparing fluorometry with other semen evaluation techniques, Bilgili *et al.* (1985b) concluded that fluorometry proves to be an excellent technique for the routine evaluation of semen and that it may replace conventional staining techniques.

2.2.5.2 Advanced methods of fowl semen evaluation
Due to the subjective nature of many of the semen evaluation techniques, the need for objective tests was identified. This need resulted in the development of a number of alternative techniques for the evaluation of semen. Some of these methods will be evaluated in this review.

2.2.5.2.1 *Computer assisted sperm analysis- CASA*
In poultry, as in any other species of animal, an objective evaluation of sperm is required in any breeding or research facility. The evaluation of sperm motility is often of little importance due to the low accuracy and subjectivity of tests (Iguer-Ouada & Verstegan, 2001). Presently, both conventional or manual and computer assisted sperm analysis (CASA) or automated methods have become popular in the evaluation of semen quality (Zavos *et al.*, 1996).

An instrument for assessing mammalian sperm quality, the Sperm Quality Analyzer®, has been validated for the use of evaluating avian semen samples (McDaniel *et al.*, 1997). The Sperm Quality Analyzer® is a device which measures the sperm quality index (SQI) by

passing a beam of light through a semen sample and quantifying the amplitude and frequency of disruption of that light beam by the movement of the sperm (Zavos *et al.*, 1996; McDaniel *et al.*, 1998; Parker *et al.*, 2000; Parker *et al.*, 2002; Dumpala *et al.*, 2006). The advantages of using the Sperm Quality Analyzer® are the speed at which a reading can be taken; it requires only 40 seconds, and the simplicity of operation of the device (McDaniel *et al.*, 1998). McDaniel *et al.* (1998) found that sperm concentration, viability and motility all contribute towards the SQI and this was later confirmed by Parker *et al.* (2000) who added that the SQI was directly correlated with sperm concentration, sperm viability and fertility. However, for an accurate SQI to be obtained, avian semen needs to be diluted, as the concentration of undiluted semen is so high that the sperm are unable to move freely in the capillary tube and thus the light path is not disrupted (McDaniel *et al.*, 1998; Parker & McDaniel, 2003). Unfortunately, the SQI cannot evaluate male libido, but it can provide the broiler breeder industry, which uses natural mating, with a tool by which to place only fertile males in a breeding population (Parker *et al.*, 2000). Parker *et al.* (2002) concluded that the SQI can be used confidently as a predictor of a male's future fertilising capacity as well as a tool for adjusting insemination doses. Amann (1999) stated that at the great grandparent level, 0.1ml of semen was worth approximately $125 and thus the semen should be maximised in order to get the most chicks from the least semen. This is now possible with the use of the SQI.

2.2.5.2.2 *Sperm-egg interactions*

The ultimate test of a male's reproductive capacity is the fertilisation of eggs. However, fertilisation is a process in which both the male and female play a role. Avian male bird fertility may be assessed by the use of an assay which investigates the interaction between the male sperm and the female ovum which results in the production of a fertile egg. To avoid any female effects on the determination of male fertility, an *in vitro* sperm-egg binding assay has been developed (Barbato *et al.*, 1998) which mimics the *in vivo* process. In order for the sperm to reach the site of fertilisation, they have to survive transport from the male reproductive tract into the female reproductive tract (Wishart *et al.*, 2004), selection, transport and storage within the oviduct (Brillard, 1993; Bakst *et al.*, 1994) of the female, egg formation mechanisms such as the secretion of albumen and shell layers and the movement of the egg through the tract (Bakst *et al.*, 1994) and finally the sperm have to remain viable to complete the fertilisation process.

Upon ovulation of the ovum from the ovary, the ovum is caught by the fimbriated region of the infundibulum and guided to the ostium (Romanoff, 1960; Bramwell *et al.*, 1995). The ovum travels a few centimetres through the infundibulum to the site of fertilisation (Olsen & Neher, 1948; Romanoff, 1960; Bakst *et al.*, 1994; Barbato *et al.*, 1998). Awaiting the arrival of the ovum at the site of fertilisation is a select group of sperm. These sperm may have been introduced into the oviduct hours, days or weeks prior to reaching the infundibulum (Bakst *et al.*, 1994). Regardless of whether the ovum is fertilised or not, it travels through the oviduct undergoing egg formation procedures and is expelled from the female body (Bakst *et al.*, 1994). Within an hour of the previous oviposition, ovulation of the next mature follicle will occur (Romanoff, 1960; Bakst *et al.*, 1994), thus starting the cycle again.

2.2.5.2.2.1 Sperm transport and storage within the hen's oviduct

In order for sperm to reach the site of fertilisation (the infundibulum), the sperm have to traverse the hen's reproductive tract. Allen and Grigg (1957) found that the mechanisms of sperm transport differ on either side of the junction of the uterus and the vagina and thus the junction acts as a sperm selection mechanism. They concluded that sperm had to be motile in order to reach the uterovaginal junction, after which the spasmodic contraction of the muscle of the wall of the upper vagina and the lower uterus is mainly responsible for the movement of sperm from the uterovaginal junction to the infundibulum (Allen & Grigg, 1957). The ability to store a population of sperm within the oviduct of the female for two to three weeks (Schindler & Hurwitz, 1966) allows for a reduction in the number of copulations or the need of daily copulations to ensure the fertilisation of eggs throughout the laying period (Donoghue, 1999). Sperm are stored within the hen's oviduct in sperm storage tubules (SST), which are tubular glands found in the uterovaginal junction (Bobr *et al.*, 1964a; Lehrer & Schindler, 1969; Brillard & Antoine, 1990; Brillard, 1993; Birkhead, 1998). Secondary sperm storage glands have been found in the infundibulum (Bobr *et al.*, 1964a; Bobr *et al.*, 1964b; Bakst, 1994), but little knowledge is available on the sperm storage capacity and mechanisms of sperm survival in the infundibulum (Bakst *et al.*, 1994). Initially the SST located in the caudal end or the vaginal side of the uterovaginal junction are filled by the sperm, followed by the progressive filling of the more cranial SST (Verma & Cherms, 1966). The release of sperm from the SST is a controversial topic. It is not known if sperm are released from the SST in a continuous fashion as suggested by

Burke & Ogasawara (1969) or in an episodic fashion throughout the ovulatory cycle or, if sperm release is restricted to the time around oviposition and ovulation as found by Bobr *et al.* (1964b).

2.2.5.2.2.2 The perivitelline layer of the fowl egg

The fowl egg consists of four main parts which are the yolk, albumen, shell membranes and the shell (Rose, 1997). The perivitelline membrane separates the yolk of the egg from the albumen (Bellairs *et al.*, 1963; Kido & Doi, 1988). The perivitelline membrane or layer (PL) may be separated into two separate layers of different compositions and structures, the inner perivitelline layer (IPL) and the outer perivitelline layer (OPL) (Bellairs *et al.*, 1963; Bakst & Howarth, 1977a; Kido & Doi, 1988). Between the IPL and the OPL is a thin, granular continuous membrane (Bellairs *et al.*, 1963). The IPL is composed of a three-dimensional network of fibres and is in contact with the yolk and the OPL while the OPL consists of a varying number of sublayers, each of which is comprised of a latticework of fine fibrils and it is in contact with the albumen (Bellairs *et al.*, 1963; Kido & Doi, 1988). The difference in conformation of the two layers suggests that each layer has a distinct function. At ovulation, only the IPL is present (McNally, 1943; Bellairs *et al.*, 1963; Bain & Hall, 1969; Bramwell & Howarth, 1992) and it is this layer which the sperm hydrolise at the germinal disc during fertilisation (Bakst & Howarth, 1977a; Bramwell & Howarth, 1992).The OPL is layed down in the infundibulum (Bain & Hall, 1969; Okamura & Nishiyama, 1978) and acts as a barrier to prevent polyspermy (Bakst & Howarth, 1977b). During fertilisation, Koyanagi *et al.*, (1988) suggested that the PL promotes the acrosome reaction and that the enzymes released by the acrosome reaction, in turn, dissolve the PL.

2.2.5.2.2.3 Sperm-egg binding assays

The ability of sperm to bind to the PL of the ovum is an essential step in the fertilisation process. Those sperm which do not penetrate the ovum at fertilisation may become trapped in the OPL of the hen's egg (Bobr *et al.*, 1964b). This phenomenon has brought about the development of two main assays which assess male fertility. The first assesses the number of holes hydrolysed by sperm through the IPL (IPL holes), while the second assess the number of sperm trapped in the OPL (OPL-sperm). The quantitative relationship between fertility and the number of sperm which interact with the ovum was first demonstrated by Wishart (1987), who described a relationship between the number of

sperm per unit area of the membrane of an egg and the probability of the egg being fertile and suggested that the OPL-sperm of laid eggs represented those which surround the ovum at the time of fertilisation. This was later confirmed by Brillard and Antoine (1990) who concluded that the numbers of sperm trapped in the PL provide an accurate reflection of sperm storage in the SST. Since Wishart (1987) determined that fertility could be assessed by the number of sperm penetrating the egg, many others have confirmed this relationship (Bramwell *et al.*, 1995; Wishart & Staines, 1995; Wishart, 1997; Robertson *et al.*, 1998; Staines *et al.*, 1998; Hazary *et al.*, 2000) and expanded on it.

Wishart (1987) described a method by which OPL-sperm could be determined. This method requires the use of the dye diamidinophenylindole (DAPI) in phosphate-buffered saline (PBS) which stains sperm nuclei and requires a fluorescence microscope to visualise the blue fluorescing sperm nuclei. In order to determine IPL-holes, pieces of IPL are spread on microscope slides and examined at x100 magnification using darkground optics (Robertson *et al.*, 1997; Wishart *et al.*, 2004).

Barbato *et al.* (1998) assumed that an *in vivo* event could be mimicked *in vitro* and developed several *in vitro* assays to determine sperm-egg binding. The assays utilise microwell plates which are coated with a PL extract. Depending on the nature of the sample, a certain percentage of sperm bind to the microwell while the unbound sperm are removed by washing. The microwell is stained with DAPI which causes the sperm nuclei to fluoresce and the numbers of sperm are counted using a fluorescent microscope. Robertson *et al.* (1998) found that the numbers of sperm interacting with the IPL *in vitro* are linearly correlated with the numbers which interact with the IPL *in vivo* and furthermore that the numbers of points of hydrolysis formed in the IPL *in vitro* and thus by inference *in vivo,* are related to the proportion of fertile eggs. Staines *et al.* (1998) concluded that the assay of IPL-holes was superior to assaying the OPL-sperm due to the ease and simplicity of the former assay as well as the fact that the latter assay requires the use of DAPI which is carcinogenic. Sperm in the OPL and IPL-holes from regions other than over the germinal disc could also be used to predict fertility but with less certainty than the IPL-holes over the germinal disc (Wishart, 1997). Hazary *et al.* (2000) agreed with the conclusions made by Wishart (1997) in that, compared to other assays of sperm-egg interaction, the assay of IPL-holes over the germinal disc has the advantage of being

technically more straightforward, simpler and quicker than the assay of OPL-sperm because no staining and only simple light microscopy are required rather than fluorescence microscopy (Wishart & Staines, 1995), and it is also quicker than the assay of IPL-holes from regions other than the germinal disc, because larger numbers of the germinal disc IPL-holes are concentrated in one location. The benefit of the sperm-egg binding assay over the OPL-sperm assay and the IPL-hole assay is that the sperm-egg binding assay eliminates the effect of the hen on the determination of the fertility of an individual male (Donoghue, 1999).

Some of these objective techniques require the use of expensive equipment and thus are not readily available for use in a large sector of the poultry industry.

2.2.6 The role of hormones in male reproduction

As for most vertebrates, reproduction in the bird is controlled by the hypothalamo-pituitry-gonadal axis (Bédécarrats *et al.*, 2006) and male hormones are known to be responsible for the expression of male secondary sexual characteristics and for the promotion of spermatogenesis (McLachlan *et al.*, 1996). Many assays which measure hormone levels have been developed in the thought that they may reflect the fertility of the male bird.

2.2.6.1. Individual hormones and their functions

Male reproductive physiology is controlled endocrinologically by two gonadotrophins, namely luitenizing hormone (LH) and follicle stimulating hormone (FSH) (Hewitt, 1998). Information from the external environment, such as changes in daylength, housing conditions or social status, and from the internal environment relating to general health, nutrition and bodily maturation, is processed in the brain and causes a response in the secretion of gonadotrophin releasing hormone (GnRH) (Ottinger, 1983; Sharp & Gow, 1983) from the hypothalamus (Bédécarrats *et al.*, 2006). Small amounts of GnRH are released from the hypothalamus and function as neuroendocrine signals which are amplified by the pituitary gland (Sharp & Gow, 1983) and stimulate the synthesis and release of gonadotrophins, LH and FSH, from the anterior pituitary (Chou & Johnson, 1987; McLachlan *et al.*, 1996; Vizcarra *et al.*, 2004; Bédécarrats *et al.*, 2006). In turn, LH and FSH stimulate the development of the gonads and the activation of this axis results in

the initiation and maintenance of sperm production (Culbert *et al.*, 1977; Sharp *et al.*, 1977; Bédécarrats *et al.*, 2006).

Steroidogenesis (the synthesis of steroid hormones) occurs within, but is not limited to, the interstitial tissue of the testes, which are composed of Leydig cells in close association with blood and lymphatic vessels (Hewitt, 1998). Leydig cells are the only testicular cells which display LH receptors (Hewitt, 1998). Luteinising hormone binds to these receptors and testosterone is secreted from the Leydig cells in response to the LH stimulation (McLachlan *et al.*, 1996; Vizcarra *et al.*, 2004). Levels of LH and testicular steroids in the plasma are maintained in equilibrium by the negative feedback effects of testosterone on LH release (Ottinger, 1983; Sharp & Gow, 1983; Hewitt, 1998). The increased frequency of pulsatile discharges of LH in the absence of testosterone, observed by Wilson and Sharp (1975) in castrated cockerels is evidence that testicular steroids (testosterone) act directly on the hypothalamus to regulate the pulsatile release of GnRH and thus LH (Sharp & Gow, 1983; Hewitt, 1998). The relationship between LH and testosterone is one of association. It was found by Vizcarra *et al.* (2004) that testosterone was secreted in an episodic fashion and that these pulses closely followed LH pulses. In intact cockerels, pulses of LH secretion occur every 1.5 to 3 hours and their frequency and amplitude are directly related to the mean level of plasma LH (Sharp & Gow, 1983).

Follicle stimulating hormone is needed for the stimulation of Sertoli cell division, maturation, secretory capacity and cytoskeleton arrangement (McLachlan *et al.*, 1996) as well as for the growth of the primary spermatocytes and the mitotic division involved in the production of secondary spermatocytes (Kumaran & Turner, 1949b). Vizcarra *et al.* (2004) found that circulating FSH concentrations vary significantly over the course of the day and that FSH is secreted in an episodic fashion, resulting in distinct pulses of FSH in the male fowl.

Testosterone is essential for the development of the secondary sexual characteristics, normal behaviour (Hewitt, 1998) and for the promotion of spermatogenesis (McLachlan *et al.*, 1996). The secretion of testosterone occurs both locally in the testes and into the general circulation (Harding, 1986). Peripheral circulating testosterone is needed for the maintenance of the secondary sexual characteristics, sexual behaviour and for the

negative feedback control on gonadotrophin secretion; while the local function of testosterone within the testes is to control spermatogenesis (Hewitt, 1998).

2.2.6.2 Use of hormones as indicators of fertility

Male sexual hormones have specific roles in reproduction including influencing secondary sexual characteristics, sexual behaviour and the production of male gametes. With this in mind, assays for the detection of these hormones have been developed in the hope that they would be an indicator of male fertility.

The hormone testosterone plays an essential role in spermatogenesis, particularly in spermatid maturation and in the expression of male secondary sexual characteristics (McLahlan *et al.*, 1996). Previous studies have found that circulating testosterone concentrations are higher in fertile males when compared with infertile males (Pierrepoint *et al.*, 1978) and that testosterone concentrations have a clear correlation with sperm production (Rawlings *et al.*, 1972; Sundqvist *et al.*, 1984). Lubicz-Nawrocki and Glover (1970) found that circulating testicular testosterone was required by mature epididymal spermatozoa in order to exhibit normal motility *in vitro* and to maintain fertilising capacity. Normal testosterone levels are required for male sexual characteristics such as libido and ejaculation (Smith *et al.*, 1981; de Souza *et al.*, 2004). Lunstra *et al.* (1978) found that bulls with higher testosterone levels reached puberty earlier than bulls with low testosterone concentrations and that attainment of mating ability was related to circulating testosterone concentrations. In the stallion, Berndtson and Jones (1989) found that testosterone per paired testes was positively related to sperm production per paired testes and concluded that testosterone was related in a positive manner to quantitative rates of sperm production. In castrated dogs, DePalatis *et al.* (1978) found that there were undetectable plasma testosterone concentrations. In cockerels, Driot *et al.* (1979) broke testosterone secretion up into three clearly definable phases; in young birds, plasma testosterone levels were low but in the prepubertal period, at 11 weeks of age, they started to rise and continued to rise until 22 weeks of age when adult levels were reached. Hocking and Bernard (2000) found that in male broiler breeders, plasma testosterone increased to a peak at 30 weeks of age, after which it decreased and remained relatively constant from 36 to 60 weeks and that this rise and fall in testosterone reflects the rise and decline in mating activity. This evidence supports the role of testosterone in fertility.

Many different methods have been developed which allow for the determination of the testosterone concentration within a plasma sample. However, some of these techniques require large plasma volumes and this proves to be a problem when experiments require serial sampling (Driot *et al.*, 1978). Techniques such as the direct radioimmunoassay (RIA) (Driot *et al.*, 1978) and enzyme immunoassay (EIA) (Active® Testosterone EIA, 2004) of testosterone require small sample sizes and are the more popular techniques used today.

The use of hormones as indicators of fertility has been widely discussed in the literature with arguments both for and against their use. The onset of both puberty and spermatogenesis are associated with an increase in the plasma concentration of LH and consequently an increase in the plasma concentration of testosterone (Culbert *et al.*, 1977; Sharp *et al.*, 1977). Culbert *et al.* (1977) found that plasma levels of LH rose significantly between 19-20 and 21-22 weeks of age to reach a characteristic adult level, after which significant increases in the mean concentration of testosterone occurred 1-2 weeks after the increase in the concentration of LH. Thus the increased secretion of testosterone is a result of the increase in LH and it is likely that the increased plasma LH stimulates steroid secretion by the testes of the cockerel when spermatogenesis begins (Culbert *et al.*, 1977). Sharp *et al.* (1977) described the sequence of endocrine events, found to be associated with the onset of puberty, as an increase in the total amount of LH in the pituitary gland when the testes started to enlarge and as an increase in the total amount of testosterone in the testes and in the concentration of LH in the plasma. Sharp (1975) found that birds which were poor layers or which produced the least sperm could be distinguished from others by their patterns of LH secretion during sexual development and concluded that differences in mean plasma LH concentrations in individual birds of either sex before the onset of puberty appeared to be related to subsequent reproductive performance. However, there are disagreements in the literature concerning the use of LH as an indicator of fertility. Lewis *et al.* (2005) concluded that plasma LH and FSH concentrations are of minimal use in the broiler breeder industry as they were not found to predict the age at sexual maturity or the subsequent egg production. Concentration of LH in the plasma is measured by LH radioimmunoassay (Follett *et al*, 1972; Wilson & Sharp, 1975; Sharp *et al.*, 1977; Lewis *et al.*, 2005).

Thus, LH seems to have its place as the best indicator of puberty; however, it does not have the ability to predict the age at sexual maturity or the subsequent egg production of an individual bird. Testosterone has been evaluated across species and it can be seen that there is a definite role for testosterone in fertility.

2.2.7 Physical traits as reliable indicators of male fertility

The use of male secondary sexual characteristics as indicators of fertility has been questioned. The reason for the continued interest in this field is that a highly correlated response of one or several characteristics with fertility would provide a useful tool to the broiler breeder industry to be able to select males for breeding based on a visual assessment of the secondary characteristics rather than having to run fertility trials such as semen analysis, as well as providing a factor which a geneticist could select upon when selecting potential breeding stock (Wilson *et al.*, 1979).

The phenotype-linked fertility hypothesis predicts that male sexual ornaments reflect the fertilising efficiency of the bird (Pizzari *et al.*, 2004). Sexual ornaments refer to the comb and wattle of the male fowl. The literature concerning the use of secondary sexual characteristics as indicators of fertility is controversial in that some authors have concluded that sexual ornaments do predict fertility whereas others disagree. Pizzari *et al.* (2004) said that the idea of a phenotypic relationship between male ornaments and fertilising efficiency is often tested in bird populations in which environmental effects may mask the underlying genetic associations between the male ornaments and fertilising ability. Burrows and Titus (1939) found a non-significant correlation coefficient ($r = 0.12$) between comb size and semen production and between wattle size and semen production ($r = 0.03$) and concluded that general outward appearances are not necessarily related to semen production. However, McGary *et al.* (2002) confirmed that a relationship between secondary sexual characteristics and fertility did exist in the domestic fowl in that male broiler breeders with larger combs within specific strains were likely to have a higher fertility. Bilcik *et al.* (2005) found no evident correlations between the size of the secondary sexual characteristics and sperm quality which was in agreement with Pizzari *et al.* (2004) who found no correlation between comb size and sperm quality, but they did find that the expression of a sexual ornament, namely the comb, reflects testicular mass ($p<0.05$) and thus by assumption, the rate of sperm production. Based on their findings, Pizzari *et al.*

(2004) proposed that male fertilising efficiency consists of two independent events, one being gonadal investment which can be predicted by comb size and the second being determined by sperm quality.

The link between ornaments, or secondary sexual characteristics, and fertility seems to be mediated by testosterone which is known to stimulate comb growth (Kumaran & Turner, 1949b; Zeller, 1971; Sharp, 1975; Driot *et al.*, 1979; Hagelin & Ligon, 2001) and testicular weight (Kumaran & Turner, 1949b; Driot *et al.*, 1979) and thus the growth of the comb tends to be correlated with gonad growth and weight (Breneman, 1937; McGary *et al.*, 2002).

Besides the comb, other characteristics which have been hypothesised to indicate male fertility are body weight, pelvic and leg conformation (McGary *et al.*, 2003),and tarsus and spur size (Bilcik *et al.*, 2005). McGary *et al.* (2003) found that body weight did not correlate with fertility in the two strains of broiler breeder used in the trial but that pelvic conformation could be used as a parameter to predict fertility in one of the strains, as fertility declined with an increase in dorsal pelvic width. They suggested that the latter was due to the inability of the male to transfer semen successfully to the female as an increased distance between the male and female cloacas is experienced during copulation. It was also concluded that leg size does not seem to have a direct impact on male fertility levels. Bilcik *et al.* (2005) determined that, in general, males with larger combs had wider tarsus diameters and larger spurs, however they concluded that there was an overall lack of association of these characteristics with fertility and sperm quality.

Certain characteristics do not have the ability to indicate the fertilising ability of a male fowl. These characteristics include the tarsus size, size of the spurs, leg size and body weight. Comb area does have some possibility of being a good predictor of fertility, as does pelvic conformation. Although ornaments which are testosterone dependent vary with testicular mass and thus reflect sperm production, they do not relay information on the gametic potential of the male and thus the expression of an ornament is unlikely to reflect the overall fertilising potential of a male bird (Pizzari *et al.*, 2004).

There are a large number of techniques available for the assessment of male fertility. Most of these techniques vary, in that different aspects of fertility are being assessed. The choice of which method to use to evaluate the fertility of males should be based on the facilities available and the desired outcome. However, if roosters are to be culled for a low fertility, the decision should be based on the evaluation of a minimum of two ejaculates and never on a single ejaculate (Gill & Amann, 2002).

2.3 FACTORS AFFECTING FERTILITY WITH SPECIAL REFERENCE TO LIGHTING

Broiler breeder fertility may be affected positively or negatively, by a number of different variables such as disease, stocking density, feeding regime and lighting procedures (Edens, 1983; Aviagen, 2001; Ciacciariello, 2003; Tolkamp *et al.*, 2005). In order to maximise the fertility of broiler breeders, proper management procedures need to be practiced. The poultry breeder has a considerable amount of information available concerning the effects of different managerial practices on poultry fertility (Bajpai, 1963). Even though all aspects of management are important for maximising fertility, this review will focus on lighting techniques for broiler breeders.

2.3.1 Physiological response to light

The pathway for the transportation of photoperiodic information to the brain, in order to modify the behavioural and sexual activity of poultry, differs in some aspects to that found in mammals (Sharp, 1993; Lewis & Morris, 2006). The eye transduces energy from photons by photosensitive pigments in rods and cones and transmits the signal to the brain through neurons, where the signal is integrated into an image (Etches, 1996). However, for the purposes of reproduction, the perception of light does not depend on the photoreceptors in the eye. The second route is via the pineal gland (Sharp, 1993; Lewis & Morris, 2006). In birds, the pineal gland is located in the triangular space between the cerebral hemispheres and the cerebellum on the top of the brain (Lewis & Morris, 2006). Due to the location of the pineal gland, light is able to pass directly through the skull and cranial tissues to reach the gland (Lewis & Morris, 2006), although it does not seem to play an essential role in the transduction of photoperiodic information to the brain (Sharp, 1993). The extraretinal photoreceptor mediating the photoresponse of birds is found in the hypothalamus (Sharp, 1993; Lewis & Morris, 2006). Light which penetrates directly

through the skull and tissues reaching the extraretinal photoreceptor controls the secretion of GnRH which in turn travels to the anterior pituitary where it stimulates the release of LH and FSH (Lewis & Morris, 2006). In spring, increasing photoperiods trigger the secretion of LH and FSH, which in turn results in the development or maturation of the gonads (Deviche, 1983; Nicholls *et al.*, 1988).

It is of both practical and theoretical concern to know what increase in photoperiod is needed to result in the secretion of gonadotrophins and gonadal growth (Etches, 1996). In the domestic chicken, plasma LH concentrations are directly related to different patterns of photostimulation (Dunn & Sharp, 1990). Dunn and Sharp (1990) concluded that concentrations of plasma LH in the domestic chicken could be used to establish that there is a range of photoperiods, within which there is a direct relationship with the photoinduced activation of the reproductive function. The authors further concluded that the shortest photoperiod needed to stimulate LH release (critical daylength) was less than 10.5 hours and the shortest photoperiod needed to stimulate the maximum release of LH (saturation daylength) was between 10.5 and 12.75 hours for dwarf broiler breeders.

2.3.2 Lighting programmes for broiler breeders

The problem faced by the poultry industry concerning lighting techniques is that most of the information available describes the effects of photoperiodicity on either the egg production of hens (Bajpai, 1963) or with testes change in wild birds (Parker & McCluskey, 1964). The male broiler breeder has received very little attention (Parker & McCluskey, 1964; Lewis, 2006) and thus a limited amount of data is available concerning the effect of light on the male broiler breeder (Bajpai, 1963; Sexton, 1983; Lewis, 2006). This is a serious concern considering the fact that male and female broiler breeders are usually subjected to the same photoschedules during rearing (Sexton, 1983; Renden *et al.*, 1991; T. Nixon, 2008, Pers. Comm., Meadow Feeds, P.O. Box 426, Willowton, Pietermaritzburg, 3200).

Lighting for broiler breeders is complicated, as they cannot be thought of or treated as big chickens (Lewis & Morris, 2006). The lighting programmes recommended for broiler breeders are similar to those used for laying hens (Ciacciariello & Gous, 2005; Lewis,

2006). These programmes usually consist of an 8 hour photoperiod during the rearing phase, followed by a transfer to 11 or 12 hours at around 20 weeks of age and then a weekly series of increments until a maximum of 15 or 16 hours at 23 to 25 weeks of age is reached (Aviagen, 2001; Lewis, 2006). However, the problem arises in that, unlike laying hens, broiler breeders are seasonal breeders (Lewis & Morris, 2006) and thus the broiler breeder's response to light is strongly influenced by two factors, namely restricted feeding and photorefractoriness (Lewis, 2006).

2.3.2.1 Restricted feeding

Meat producing birds are primarily selected for rapid growth or body weight gain, feed conversion efficiency and body conformation (Fontana *et al.*, 1990; Aviagen, 2001; Lewis, 2006). As a result of this increase in growth, the feed intake and body weight gain of the broiler breeder needs to be controlled during the rearing period, in order to prevent a decline in fertility due to the negative genetic correlation between growth and fertility, poor body conformation and even mortality (Sexton, 1983; Fontana *et al.*, 1990; Aviagen, 2001; Ciacciariello & Gous, 2005; Cobb, 2005; Lewis, 2006). To achieve optimum fertility, the birds are grown to the target bodyweight-for-age by maintaining accurate control of the birds' growth through sample weighting of random birds in a flock and the consequent adjustment in feed allocation (Aviagen, 2001).

2.3.2.2 Photorefractoriness

Photorefractoriness is a natural condition that prevents animals from becoming sexually active when the environmental conditions are inappropriate for the raising of offspring (Farner *et al.*, 1983; Nicholls *et al.*, 1988; Lewis, 2006) or put simply, it is the inability to respond photosexually to an otherwise stimulatory daylength (Siopes, 1984; Nicholls *et al.*, 1988; Siopes, 1997; Lewis *et al.*, 2003; Lewis & Morris, 2006). Two forms of photorefractoriness are evident; a juvenile form and an adult form. In the juvenile form of photorefractoriness, wild seasonal breeding birds are hatched photorefractory and thus are prevented from becoming sexually mature within their first year after hatching, even though they may be somatically mature (Lewis *et al.*, 2003; Lewis, 2006; Lewis & Morris, 2006). The adult form of photorefractoriness induces a moult, terminates nesting behaviour and causes gonadal regression, thus ending reproduction in sexually mature birds (Lewis *et al.*, 2003; Lewis, 2006; Lewis & Morris, 2006). This prevents offspring from

being raised under unfavourable environmental conditions (Farner *et al.*, 1983). Once the adult bird is photorefractory, it will remain so until the following years breeding season (Lewis & Morris, 2006). In wild birds, adult photorefractoriness may be expressed in a number of different forms, namely absolute, relative and opportunistic photorefractoriness (Dawson & Sharp, 2007). Species with short breeding seasons generally become absolutely photorefractory in which spontaneous gonadal regression occurs in response to long photoperiods followed by no response when exposed to further increases in daylength (MacDougall-Shackleton *et al.*, 2006; Dawson & Sharp, 2007). Species which have long breeding seasons may become relatively photorefractory whereby long photoperiods induce the photorefractory state but a decrease in photoperiod needs to be experienced in order to induce gonadal regression which is reversed by an increase in photoperiod at any time (Sharp, 1996; Dawson *et al.*, 2001; Dawson & Sharp, 2007). In opportunistic breeders, rapid gonadal maturation is permissible over a large proportion of the year (Dawson & Sharp, 2007) and these species are able to terminate gonadal regression and initiate gonadal recrudescence with exposure to very long daylengths (MacDougall-Shackleton *et al.*, 2006). These three states may not be mutually exclusive as there is a graduation between the extremes (MacDougall-Shackleton *et al.*, 2006) for example, during the initial phase of absolute photorefractoriness there is a brief period during which the gonads regress in advance to the decrease in GnRH and this period may be equivalent to relative photorefractoriness (Dawson & Sharp, 2007). In turkey hens it is thought that relative photorefractoriness is a lesser form of, and precedes, absolute photorefractoriness (Proudman & Siopes, 2002; Proudman & Siopes, 2004). During the breeding season, neuroendocrine changes cause the hen to become unresponsive to changes which initially stimulated reproduction leading to the hen becoming relatively photorefractory, cessation of lay occurs if the photoperiod is reduced, leading on to absolute photorefractoriness and ceasation of lay even though the photoperiod does not change (Proudman & Siopes, 2004). Proudman and Siopes (2002) found that in a flock of turkey hens, a range of seasonal reproductive behaviour was evident with some hens displaying relative photorefractoriness, others absolute photorefractoriness and some extended periods of photosensitivity. Proudman and Siopes (2006) reported that turkey hens which were relatively photorefractory could be separated from those which were absolutely photorefractory by a change in photoperiod applied at 48 weeks of age (the hens were maintained on 18 h). Hens which ceased laying after the photoperiod was

changed to 13 h and returned to lay when the 18 h photoperiod was resumed were classified as relatively photorefractory but the hens which did not return to lay after 4 weeks of being on 18 h were deemed to be absolutely photorefractory. No information is available concerning the relative form of photorefractoriness present in broiler breeders. Thus, photorefractoriness is an important topic for producers as it is a major mechanism that restricts the continuous production of eggs and semen (Siopes, 1997).

The primary environmental feature used by birds to time reproduction or the breeding season, is the annual change in daylength (Nicholls *et al.*, 1988). Long photoperiods have two distinct yet opposite effects on the reproductive system, photostimulation or initiation of reproductive development and the induction of photorefractoriness (Nicholls *et al.*, 1988; Dawson *et al.*, 2001). Dissipation of photorefractoriness leading to the attainment of full gonadal function requires an effect of short photoperiods, recovery of photosensitivity, which is then followed by an effect of long photoperiods, which initiates photostimulation (Dawson, 2001). The results from an experiment conducted by Dawson (2001) using starlings, showed that adult photorefractoriness is not a process which begins some time after photostimulation and then causes gonadal regression, nor is it a consequence of photostimulation on the reproductive tract. Rather, it is a process that appears to begin during the first long photoperiod. The fact that photostimulation results in the maturation of the reproductive tract which is then later caused to regress through photorefractoriness, means that photostimulation and photorefractoriness must have differences in the rate at which they reach completion (Dawson, 2001).

Researchers have put forward many different methods by which photorefractoriness may be controlled. In mammals, seasonal changes in reproduction occur in response to the changes in the secretion of melatonin by the pineal gland (Juss, 1993). The results from an experiment involving the administration of melatonin to quails showed that testicular growth was not prevented by the administration of melatonin during stimulatory photoperiods and it was concluded that melatonin was not responsible for the control of reproduction in birds (Juss, 1993). Prolactin is another possible endocrine candidate for the cause of photorefractoriness (Juss, 1993). When investigating starlings, Dawson and Goldsmith (1983) found that increased prolactin secretion was associated in some way with the onset of photorefractoriness but they were unclear as to whether the increase in

prolactin secretion was a cause or consequence of photorefractoriness, or simply of gonadal regression. In 1998, Dawson and Sharp concluded that the seasonal peak of photoinduced prolactin secretion, associated with the onset of adult photorefractoriness, accelerated gonadal regression, but was not required for the eventual development of photorefractoriness. Proudman and Siopes (2002) confirmed that prolactin did not assure the expression of photorefractoriness in turkey hens. A number of studies support the role of thyroid hormones in initiating and maintaining photorefractoriness in birds (Dawson *et al.*, 1986; Nicholls *et al.*, 1988; Dawson, 1989; Siopes, 1997; Dawson *et al.*, 2001; Yoshimura *et al.*, 2003). Two different methods have been used to study this hypothesis. Thyroidectomy has been the direct approach to studying the involvement of the thyroid with photorefractoriness in that reproductive performance and the development of photorefractoriness can be monitored after the removal of the thyroid gland (Siopes, 1997). The problem with this method arises with the fact that different bird species respond differently to a thyroidectomy in that there are species differences in the exact effects of thyroid hormones on seasonal changes of the reproductive capacity of the bird (Nicholls *et al.*, 1984; Ball, 2006). In starlings, thyroidectomy does prevent the progression towards and attainment of a photorefractory state (Follett & Nicholls, 1984; Dawson, 1989) yet in sparrows it would appear that a thyroidectomy might actually cause photorefractoriness (Dawson, 1989). Studying photorefractoriness in turkey hens by thyroidectomy is not suitable as the hen requires the presence of the thyroid gland to initiate and maintain ovarian function and in the absence of egg production, a complete evaluation of photorefractoriness cannot be made (Siopes, 1997). The removal of the thyroid gland could disrupt a wide number of metabolic processes and the resulting effect on the reproductive system may then be indirect rather than direct (Dawson *et al.*, 1986). An indirect approach to assessing the role of the thyroid gland is through the use of exogenous hormones. Results from an experiment performed with starlings showed that the presence of thyroxine, in association with long days, causes the processes leading to photorefractoriness to begin, but that photorefractoriness is not driven by thyroxine (Dawson *et al.*, 1989). The absence of thyroxine totally prevented the onset of photorefractoriness and thus concluded that the presence of thyroxine is simply needed for the process of photorefractoriness and the bird to respond appropriately to ambient daylength (Dawson *et al.*, 1989; Dawson *et al.*, 2001). Siopes (1997) suggested that in turkeys, the thyroid gland could be as important as light in sustaining photorefractoriness.

Proudman and Siopes (2002) suggested that the role of the thyroid in initiating photorefractoriness is less consistent in the turkey than it is in wild birds. This may be due to the turkey hen developing mechanisms that inhibit the driving factors for photorefractoriness, possibly brought about through strong genetic selection for egg production (Proudman & Siopes, 2002). By treating turkey hens with an anti-thyroid compound such as 6-n-propyl-2-thiouracil, photorefractoriness can be terminated due to the lack of thyroid hormones (Siopes, 1997). However, the mechanism by which the onset of photorefractoriness is controlled has so far remained elusive (Nicholls *et al.*, 1988; Juss, 1993; Dawson, 2001) and further research is required in this field of study.

Two lighting techniques are used to interrupt the refractory state of normally photorefractory species (Woodard *et al.*, 1986). The dissipation of photorefractoriness occurs in birds, not on restricted feeding, by exposure to around two months of non-stimulatory photoperiods (Lewis & Morris, 2006). In nature, these are presented in the form of short winter daylengths (Lewis & Morris, 2006) or artificially, when the bird is exposed to short photoperiods (Wolfson, 1958; Nicholls *et al.*, 1988; Dawson, 2001; Ciacciariello & Gous, 2005). Short photoperiods have the effect of restoring the hypothalamus to a state in which it is ready to function by causing a renewal of synthesis or a considerable increase in the rate of synthesis of GnRH (Dawson *et al.*, 1986). Lewis *et al.* (2004) suggested that ≤ 11 hour photoperiods dissipated juvenile photorefractoriness in broiler breeders more rapidly than ≥ 12 hour photoperiods and that this might indicate the changing point from a non-stimulatory to a stimulatory photoperiod. The second lighting technique available to terminate photorefractoriness is by a reduction in illuminanace for birds maintained on stimulatory daylengths or long days (Siopes, 1984; Woodard *et al.*, 1986). The termination of photorefractoriness by low light intensity probably occurs due to the light stimulus falling below the threshold of the photoreceptors mediating the photosexual response, and as a result, the bird would effectively be exposed to darkness, thereby changing the bird's state from photorefractory to photosensitive (Siopes & Wilson, 1978; Siopes, 1984). In chukar partridges, Siopes and Wilson (1978) found that small changes in light intensity have little effect in terminating photorefractoriness. In turkey hens, Siopes (1984) found that the light intensity required to terminate photorefractoriness was as low as 0.5 lux but not ≥ 2.2 lux. After reviewing literature concerning illuminance, Lewis *et al.* (2003) suggested that in order to completely dissipate adult

photorefractoriness in birds maintained on long days, the illuminance needs to be substantially less than 1 lux.

2.3.2.3 Photoperiods and sexual maturity

The lighting techniques recommended by breeding companies vary depending on the type of house in use. In closed houses, the use of short days throughout rearing followed by an increase in daylength around 20 weeks of age is recommended for broiler breeders (Aviagen, 2001; Gous & Cherry, 2004; Cobb, 2005). Due to the differences in daylength experienced throughout the year in different hemispheres and at different latitudes, lighting programmes recommended for broiler breeders raised in open housing becomes complicated (Gous & Cherry, 2004). Breeding companies recommend that broiler breeders not be reared in natural daylight or open houses unless the natural daylength is small, but if rearing does occur under these conditions, then a stimulus of artificial light is provided at the beginning and end of the natural day light period to provide a period of increasing daylength at 140 days of age (Cobb, 2005).

If broiler breeders are exposed to a constant photoperiod from soon after hatching, sexual maturity is achieved about one day earlier for each one hour increase in photoperiod up to 10 hours (Lewis, 2006). Sexual maturity is markedly delayed (by 3 to 4 weeks) when broiler breeders are exposed to rearing photoperiods between 10 and 13 hours with earliest sexual maturity being achieved by providing 10 hour daylengths and the latest when birds are reared on constant 13 or 14 hour daylengths (Lewis *et al.*, 2004; Lewis, 2006; Lewis & Morris, 2006). This noticeable difference in age at sexual maturity is in agreement with results found by Dunn and Sharp (1990) who determined that the shortest photoperiod needed to stimulate LH release in dwarf broiler breeders was < 10.5 hours and the shortest photoperiod needed to stimulate maximum LH release was between 10.5 and 12.75 hours in birds reared on 8 hour daylengths. These differences in age at sexual maturity for birds held on constant photoperiods, which vary in their degree of stimulatory effect, are probably a reflection of the different rates at which photorefractoriness is dissipated in broiler breeders (Lewis *et al.*, 2004). The results obtained by Gous and Cherry (2004) confirm this as they report that photorefractoriness can be used to explain

why birds held on constant 17 hour photoperiods, and not exposed to a period of short days, reached sexual maturity later than birds reared on a short daylength of 8 hours.

A prepubertal increase in photoperiod can be given to control sexual maturity (Lewis and Morris, 2006). Before photorefractoriness has been dissipated, the bird will respond to changes in photoperiod as if it had been maintained on the final photoperiod from hatch (Lewis and Morris, 2006). Lewis *et al.* (2003) found that birds maintained on constant 16 hour photoperiods, and the majority of birds transferred from 8 to 16 hour photoperiods at 67 days, had a similar mean age at first egg, which indicates that the birds given the changing photoperiod did not respond to the change but rather matured as if they had always been raised on 16 hours of light. Pullets transferred from an 8 hour photoperiod to a 16 hour photoperiod at younger than 10 weeks of age will mature approximately 3 weeks later than pullets maintained on a constant 8 hour photoperiod. However birds photostimulated at 18 weeks of age will experience an advance in maturity of about 5 weeks (Lewis *et al.*, 2003; Lewis & Morris, 2006). Gous and Cherry (2004) found that broiler breeders reared and maintained on constant 17 hour photoperiods reached sexual maturity 27days later than birds photostimulated at 19 weeks of age.

2.3.3 Effects of light on male broiler breeder performance
The effects of lighting on male broiler breeder performance are of importance as they will contribute significantly to the fertility and hatchability of a flock of broiler breeders. In most commercial facilities, male and female broiler breeders are reared together under the same lighting schedules (Brake, 1990; Renden *et al.*, 1991; Aviagen, 2001). There is a large amount of literature available concerning the effect of photoperiods on female broiler breeders, however, there is a dearth of information regarding the response of the male broiler breeder to lighting.

Parker and McSpadden (1943) observed that Rhode Island Red males displayed seasonal differences in volume, density and concentration of semen and in the total number of sperm produced. The authors concluded that sperm production by the male chicken is influenced by an annual cycle similar to that seen in egg production. Lamoreux (1943) realised that no data existed concerning the effects of different amounts of illumination on the production of semen in the male chicken. After conducting several experiments in

which males were exposed to 1, 9, 12, 14, 16, 24 hours of light per day, it was concluded that photostimulation of mature males (approximately 32 weeks of age) occurred within 4 weeks and semen production required 9 to 12 hours of light per day with males on these treatments producing greater yields of semen than males provided with 1 hour of light. Lamoreux (1943) however did not determine the effect of the photoperiods on the quality or fertilising ability of the semen produced. Bajpai (1963) found that increasing the daylength from 12 to 16 hours caused an improvement in both semen quality and quantity in year-old Rhode Island Red roosters. However, decreasing the photoperiod from 12 to 8 hours led to a deterioration of semen quality (measured as motility, percent live sperm, partial stained and abnormal sperm) but also to an increase in both semen volume and total number of sperm per ejaculate. Parker and McCluskey (1964) showed that sexually mature male chickens can produce adequate volumes of semen while being exposed to as little as 1 hour of light. The authors furthered this research and determined that the age of the bird at the onset of semen production was affected by the length of the daily light period during rearing, in that the birds receiving 13 hours of light produced semen before the birds receiving 1, 3 or 9 hours of light (Parker & McCluskey, 1965). Semen was first produced by one of the males exposed to 13 hours of light but the 9 hour photoperiod resulted in all the males producing semen first. The results indicate that rearing male chickens on photoperiods ranging from as little as 1 hour of light to 13 hours had no effect on the males subsequent reproductive capacity and all males were fertile by 24 weeks of age. Ingkasuwan and Ogasawara (1966) found that a 14 hour photoperiod stimulated gonadal development at an earlier age compared with an 8 hour photoperiod; however, with advancing age, the stimulatory effect disappeared. An 8 hour photoperiod was related to a delay in the initiation of the rapid phase of testicular growth compared to a 14 hour photoperiod but the testes were not prevented from obtaining normal size. Upon full testes development, the birds on the 8 hour photoperiod were capable of producing semen of a better quality than the males on the 14 hour photoperiod. The results reported by Siegel *et al.* (1969) confirmed those of Ingkasuwan and Ogasawara (1966) but showed, in addition, that fertility was not significantly affected by different daily light cycles (constant 14 hours compared with a 6 hour photoperiod with weekly 3% increases in photoperiod) used either during the growth period or the period of semen production. The authors concluded that if increasing light regimes are necessary for the production of eggs in broiler producing flocks, difficulties associated with male fertility should not be experienced. Proudfoot

(1981) produced results which disagreed with those of Parker and McCluskey (1964) in that a 2 hour daily photoperiod was found to be insufficient for stimulating maximum semen motility and fertility. After comparing the fertility of males exposed to photoperiods of 2, 6, 14 and 15.5 hours from 168 to 357 days of age, Proudfoot (1981) concluded that it is advantageous to use a 6 hour photoperiod compared with longer photoperiods, even though 6, 14 and 15.5 hours of light all supported optimum fertility for meat parent males. This is because it not only reduces the consumption of light energy, but also reduces aggressive behaviour among birds housed together. In broiler breeder cockerels, an increase in the photoperiod to 16.5 hours did not significantly affect reproductive traits except in the number of completed matings and the percent packed sperm volume (Harris *et al.*, 1984). Compared to the males on the 15.5 hours of light, the male broiler breeders on 16.5 hours had significantly fewer completed matings (Harris *et al.*, 1984). However, when considering the strain of broiler breeder used, Strain 1 males on 16.5 hours produced significantly more sperm at 35 and 40 weeks of age than the males on the 15.5 hour photoperiod (Harris *et al.*, 1984).

In turkeys, total sperm produced during the reproductive period of males exposed to long-day lighting at an early age was almost twice as great as that in birds given short-day lighting until the increase to long-day lighting at 29 weeks of age, which is a traditional lighting programme for male turkeys (Kurginshi Noonan & Bacon, 2000). Brake (1990) determined that the threshold for initiating male stimulation is between 8 and 10 hours when males are reared on an 8 hour photoperiod and thus male broiler breeders raised on an 8 hour photoperiod and given an increase of 2 hours of light at 18 weeks of age, will experience an increase in fertility. The author also concluded that male broiler breeders may be photostimulated to maturity and exhibit increased fertility without negatively affecting the female reproductive performance when the males and females are grown in the same light environment. Renden *et al.* (1991) concluded that in male broiler breeders on constant photoschedules, the maximum rate of sexual maturation would occur when the birds are exposed to 10 hours of light per day. It was also found that the earliest maturity occurred with 4 to 8 hour photoperiods and semen concentration was increased with deceasing hours of light (24 hours to 2 hours). The other major advantage of having short photoperiods would be the increased percentage of males producing semen upon artificial ejaculation, as found by Renden *et al.* (1991). Contradictory to the relationship

between photoperiod and semen concentration, the study found that testosterone levels increased after 16 weeks of age and there was a positive relationship between the levels of testosterone and the hours of light.

When designing a management programme for male broiler breeders, many factors need to be considered. Sexual maturity is known to be influenced by both body weight and exposure to light. To maximise the fertility of male broiler breeders, producers need to consider a restricted feeding programme which allows the bird to grow according to a set bodyweight-for-age growth curve. Along with this, photorefractoriness needs to be taken into account.

2.4 SUMMARY

The fertility of the male broiler breeder is of utmost importance to the poultry industry as it is a key factor in the production of viable offspring. Fertility is dependent on the correct structure and functioning of the male reproductive tract in association with the production and secretion of the male hormones. The production of high quality sperm is under the control of the male reproductive system but it can also be affected by a number of factors which include the frequency of collection, the age of the bird and the size of the testes.

The fertility of the male may be assessed by a number of different methods. These methods can be classified into two categories, namely the principal parameters and the advanced methods of semen evaluation. The principal parameters of fowl semen analysis usually include an evaluation of the volume, colour, pH, motility, morphology and percentage of live and dead sperm in the semen sample. Generally the principal parameters of semen evaluation rely on the use of light microscopy and human assessment. Here, the problem arises with the fact that the results may become subjective. In order to eliminate this subjectivity, advanced methods of semen evaluation were developed. Some of these methods include sperm-egg binding assays and the use of computers in CASA.

An alternative approach to assessing fertility, rather than through the use of a semen sample, is through the evaluation of hormone levels or secondary sexual characteristics. The use of hormones as indicators of fertility has been widely discussed in the literature

with arguments both for and against their use. The evidence provided in this review supports the role of testosterone in fertility. Certain physical characteristics or secondary sexual characteristics do not have the ability to indicate the fertility of a male bird; these include the tarsus size, size of the spur, leg size and body weight. Pelvic conformation and comb size do have some reliability as assessments of male fertility. Ornaments which are testosterone dependent vary with testicular mass and thus reflect sperm production but they do not relate any information concerning the gametic potential of the male bird. Thus secondary sexual characteristics may reflect sperm production but it is unlikely that they represent the overall fertilising potential of the male broiler breeder.

Currently, there are a wide number of fertility assessments available to the poultry industry. The choice of test used to assess the male broiler breeder's fertility will be based on the required outcome, the facilities and available budget.

Male broiler breeder fertility can be managed by a number of different practices such as feed restriction to control the growth curve of the bird as well as lighting programmes. The major factor which needs to be considered when lighting programmes are being designed for broiler breeders is photorefractoriness. Photorefractoriness has two forms, the first or juvenile form, is a condition which prevents a bird from breeding within its first year and the adult form which prevents a bird from producing offspring when the environmental conditions are not adequate. Researchers have rejected melatonin as an activator of photorefractoriness, but roles for prolactin and thyroid hormones are evident. However, the mechanism by which photorefractoriness is controlled still remains elusive and further research is required in this field. Two methods have been widely used to eliminate photorefractoriness and these include the use of exposure to short days and low light intensity. Drugs which induce hypothyroidism can also be used to dissipate photorefractoriness.

Depending on the hemisphere and the latitude at which a poultry farm is located, in combination with the type of house in use; broiler breeder companies recommend different lighting programmes for different situations. Sexual maturity can be advanced or delayed depending on whether the birds are reared on short or long days and the distinct difference in age at sexual maturity for birds reared on constant daylengths are more than

likely an indication of the different rates of dissipation of photorefractoriness. When considering changing photoperiods before photorefractoriness has been dissipated, birds will respond to the final photoperiod as if they have had been reared on it from hatch and age at sexual maturity will be affected by the age at photostimulation.

Compared to the amount of data available concerning the effects of light on female production, there is a very limited amount of information available pertaining to the effects of light on male broiler breeders. It seems that males are able to produce semen when exposed to as little as 1 to 3 hours of light but in order to obtain maximum fertility, males should be given a minimum of 8 hours of light in rear. It also seems evident that males and females can be reared under the same lighting conditions without experiencing any detrimental effects on the fertility of either sex.

The production of viable offspring in the poultry industry requires that both the male and female birds have high fertility. Current research seems to have focused more on the female broiler breeder than on the male and it is essential that more research be directed towards the male if high yields are to be achieved by the industry.

CHAPTER 3

THE EFFECTS OF DEGREE OF PHOTOSTIMULATION ON AGE AT SEXUAL MATURITY AND FERTILITY IN MALE BROILER BREEDERS

3.1 INTRODUCTION

Lighting programmes for broiler breeders reared in closed houses are similar to those used for laying hens: an 8 hour (h) photoperiod during the rearing phase, transfer to 11 or 12 h at around 20 weeks, followed by a subsequent series of weekly increments until a maximum of 15 or 16 h is reached by 23 to 25 weeks of age (Lewis, 2006). However, broiler breeders are known to display photorefractoriness. Dunn & Sharp (1990) found that for dwarf broiler breeders at 8 weeks of age the critical daylength and the saturation daylength were < 10.5 and between 10.5 and 12.75 h respectively. Lewis *et al.* (2008) more accurately determined the critical and saturation daylengths in female broiler breeders as 9.5 and 13 h respectively. Thus, an 8 h photoperiod is appropriate during rearing of broiler breeders as is a transfer to 11 or 12 h at 20 weeks, but a photoperiod of 15 or 16 h is unnecessary as this is past the saturation daylength and may accelerate the onset of adult photorefractoriness (Lewis and Gous, 2006). To prevent this, the initial increment needs to be a photoperiod that is slightly above the critical daylength, whereas, the final photoperiod, if different, needs to be shorter than the saturation daylength.

In most broiler breeder production systems, males and females are reared under the same lighting conditions (T. Nixon, 2008, Pers. Comm., Meadow Feeds, P.O. Box 426, Willowton, Pietermaritzburg, 3200). However, there is a dearth of information regarding the effects of photoperiod on male broiler breeder production as there are very few data sets available regarding this topic (Lewis, 2006). Brake (1990) suggested that broiler breeder males appear to have a lower threshold for photostimulation than females, however this conclusion was based on an experiment where only two photoperiods were tested.

The aim of this trial was to identify the effect of photostimulation applied at 20 weeks, on age at sexual maturity (ASM) and the subsequent fertility of male broiler breeders. This knowledge would allow for the design of lighting programmes that optimise male fertility

during peak egg production in females and delay the decline in fertility observed at the end of the production period.

3.2 MATERIALS AND METHODS

One hundred and forty four, 15 week old, commercially reared breeder males (female-line Ross 308 grand-parent stock; Ross Poultry Breeders Ltd., Meyerton, South Africa) from light-tight facilities (raised on 9 hours) were housed on litter in 12 light-tight rooms (two suites of 6 rooms each), with each room subdivided into 12 individual wire cages (60cm wide x 44cm deep x 60cm high).

In order to ensure that variation in body weight was kept to a minimum, birds were weighed upon arrival and randomly placed in cages. An analysis of variance confirmed that there were no significant differences in average body weight between rooms.

All birds were feed restricted and received a commercial feed of broiler breeder grower pellets. Each bird received 120g of feed per day. Birds were provided with *ad libitum* access to water.

During the period of acclimatisation (housing to photostimulation), all rooms were given an 8 h photoperiod (07.00-15.00). A 60W incandescent lamp, located 1.8m above the floor, was used in each room to provide illumination that had a mean illuminance of 47±1.8 lux. At 20 weeks of age, photoperiods were increased in each room, apart from the non-photostimulated controls in room 1, as detailed in Table 3.2.1. Due to the layout of the facilities, each treatment was represented in only one room. The aim of this experiment was to observe the effect of photostimulation, expanding on the work of Dunn and Sharp (1990) where too few treatments did not allow an accurate idea of the critical and saturation daylengths, therefore the maximum number of lighting treatments was applied to determine a more accurate regression curve forgoing replication in the process. This was considered the best treatment design as a regression curve rather than an analysis of variance is the more appropriate statistical analysis.

Table 3.2.1 *Schedule for lighting treatments at 20 weeks of age*

Room	Photoperiod (h)	Lights on-off
1	8.0	07:00-15:00
2	9.5	07:00-16:30
3	10.5	07:00-17:30
4	11.5	07:00-18:30
5	12.5	07:00-19:30
6	14.0	07:00-21:00
7	9.0	07:00-16:00
8	10.0	07:00-17:00
9	11.0	07:00-18:00
10	12.0	07:00-19:00
11	13.0	07:00-20:00
12	18.0	07:00-01:00

To determine testosterone levels, 1-2mls of blood was drawn from the brachial vein of 5 birds in each room. Samples were collected at 19 weeks of age to determine a baseline testosterone level, after which samples were collected from the same birds at 23, 25 and 30 weeks of age. The samples were transferred to the laboratory for centrifugation at 3000rpm for 15 minutes. The sera were extracted and stored in plastic mailing tubes and frozen. The sera were later analysed using an enzyme immunoassay kit[2] which has been validated for use in poultry (Mohan *et al.*, 2002).

Digital photographs were taken of the left and right-hand side of the head of each bird in order to determine the area of the comb (McGary *et al.*, 2003). Each picture contained a centimetre ruler for image calibration. The computer mouse was used to trace the perimeter of the comb in the digital photographs and the comb area was calculated using the image analysis software *Image J* (Rasband, 2006). Photographs were taken at 22, 23, 24, 25, 26, 30 and 51 weeks of age. The comb area was used to calculate the predicted testis weight (Appendix 1) of the birds (Tyler & Gous, 2006).

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[2] DSL-10-4000 Active ® Testosterone enzyme immunoassay kit from Diagnostic Systems Laboratories, Inc. 445 Medical Center Blvd. Webster, Texas 77598-4217 USA

The primary wing feathers on both the left and right wings were counted and recorded as the number of rounded and sickle feathers (Figure 3.2.1). The feathers were counted at 23, 25, 30, 37, 39, 41 and 52 weeks of age.
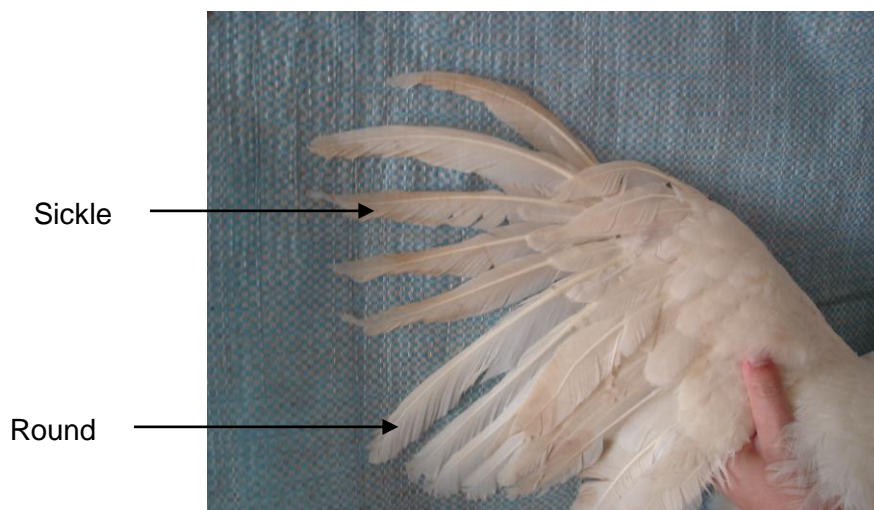
Figure 3.2.1 *Photograph showing the primary feathers of the wing and in particular, the sickle and rounded feathers*

Semen training was performed on the birds twice weekly starting from 15 weeks of age, and the date of the first semen production was recorded as the age at sexual maturity (ASM) (McDaniel & Craig, 1959). Semen analysis was performed five times during the trial; two days prior to the corresponding reported mean ASM of females on the same treatment (data from Lewis *et al.*, 2008) (Appendix 2) as females would need to be mated 2 days before ASM in order to ensure that the first egg laid was fertile, and at 33, 40, 45 and 51 weeks of age.

Semen collection was performed using a modified version of the method described by Lake (1957) in that one person held the bird while the other massaged the bird's back and collected the sample. Prior to semen collection, tubes containing Eosin and Nigrosin solution for calculation of concentration were prepared for each cage within a room (Appendix 3) as was Tyrodes solution (Appendix 4), a semen diluent, and 3% gluteraldehyde to fix semen for subsequent morphological assessments.

Immediately after collection, 50µl of the sample was fixed in 3% gluteraldehyde and 50µl of the same sample was placed into the concentration tubes and gently mixed by inversion.

Concentration was calculated by pipetting 50µl of the concentration solution to fill the two sides of a haemocytometer and the numbers of sperm were counted in 20 random blocks on either side of the haemocytometer at 40x magnification using a light microscope. This was used to calculate the number of sperm per ml of semen (Appendix 5).

Sperm motility was assessed immediately after semen collection by diluting a small sample of semen with Tyrodes solution. A drop of the diluted semen was placed on a slide and examined under a light microscope at 40x magnification. The sample was evaluated for the percentage of progressively motile normal sperm, the percentage of abnormal motile sperm and the percentage of dead sperm.

The semen placed in gluteraldehyde was later assessed using a light microscope at 100x magnification with an oil immersion lens. One hundred sperm from each sample were then classified as either normal, bent, coiled, abnormal head or abnormal tail.

To evaluate the fertility of the male birds, the number of sperm trapped in the outer perivitelline layer of laid hens eggs (OPL-sperm) after artificial insemination were counted using the method described by Wishart (1987). A semen sample was collected from all birds at 40, 45 and 51 weeks of age. The concentration was calculated for each sample as described above and mixed with Tyrodes solution to ensure that all samples contained 30 million sperm per 50µl of diluted semen (Appendix 6). Two to three commercial layer hens per male were artificially inseminated intravaginally with 50µl of this diluted semen. Eggs were collected from all the hens on days 3, 5, 6 and 8 post insemination for week 40 and days 4, 6, 8 and 13 post insemination for weeks 45 and 51, and stored in a cold room. Each egg was broken open and the ova separated from the albumen through repeated decantation and rolling on clean paper towels. Using dissecting scissors, a 1cm square piece of the perivitelline layer (PL) was cut and removed from around the germinal disc. This was washed and rinsed in phosphate buffered saline (PBS) to remove any remaining yolk or albumen and spread on a glass slide. The prepared slide was then stained by

adding a few drops of a 1mg/l solution of 4,6-diamidino-2-phenylindole in PBS (DAPI) (Appendix 7). A coverslip was placed over the piece of membrane and excess liquid was drained off. The slides were placed in an aluminium container to avoid exposure to light and transported to the microscopy laboratory. The blue fluorescing, comma-shaped sperm nuclei were counted in 20 fields of view using a fluorescent microscope[3] at 10x magnification. The number of sperm counted in 20 fields of view was then converted into a number per mm^2 of membrane (Appendix 8) and scored as fertile or infertile according to two different methods. The first method was that of Wishart (1997) in which eggs with > 3 OPL-sperm per mm^2 had a 94% probability of being fertile and the second method was that of Brillard and Antoine (1990) who determined that 0.43 sperm per mm^2 of membrane were needed for 100% probability of fertility of an egg. For comparision between OPL sperm counts after artificial insemination at weeks 40, 45 and 51, the numbers of OPL sperm counted at days 3 and 5 post insemination for week 40 were averaged and considered to be a day 4 count as there was hardly a difference between the numbers of sperm trapped in the eggs on days 3, 4 and 5. If eggs were found to be infertile on day 8 post insemination, the eggs on day 13 were not analysed and assumed to be infertile.

At the end of the trial, the 5 birds in each room, from which blood had been taken for testosterone analysis, and any birds not producing semen, were slaughtered, after electrical stunning, by cutting the blood vessels in the neck. The birds were weighed, photographs of the combs were taken and the wing feathers were counted before slaughter. The birds were dissected and the testes removed, weighed and measured using vernier callipers. The presence or absence of semen in the vas deferens was recorded (Figure 3.2.2.1). Birds which did not have semen present in the vas deferens were identified and the semen records analysed in order to estimate at what point in time the birds had probably stopped producing semen.

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[3] Olympus AX70 Stereo compound light microscope
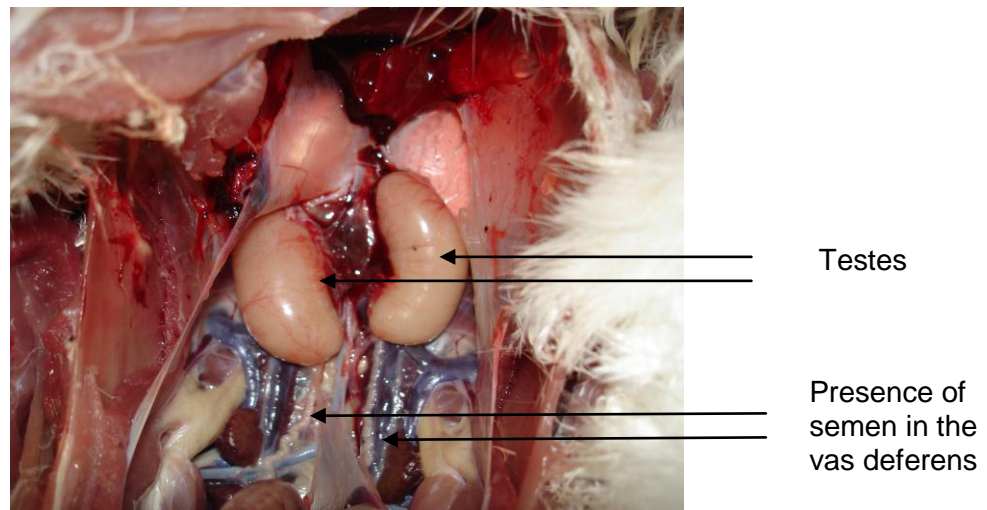
Figure 3.2.2 *Photograph showing the location of the testes in the abdominal cavity and the presence of semen in the vas deferens*

The statistical software programme *Genstat 10th Edition* (Lawes Agricultural Trust, 2007) was used to analyse the data. Due to the unbalanced nature of the data (brought about by lack of replication), it was not possible to perform an analysis of variance (ANOVA) to determine significant differences between treatments and thus regressions were used to determine responses, trends and changing points. The ASM data were analysed by standard curve regression, hinge analysis (Lewis *et al.*, 1998) and descriptive statistics. An ANOVA was performed to identify if there was a significant difference between 2 groups of males in respect to the amount of within-treatment variation in ASM as there appeared to be a visible difference. Bodyweight data was subjected to simple and multiple regression analysis. The testosterone data were subjected to standard curve regression analysis, correlations and descriptive statistics. A students *t*-test was used to compare predicted testes and actual testis weights at slaughter, as well as the left and right testes weights. It was found that the left and right testes were only significantly different for birds on 4 treatments and, considering that a semen sample is the combination of semen produced by both testes, the mean was thus used for further analysis. However, standard curve regression was performed on the left and right testis data separately. The mean testis weights were analysed using standard curve regression analysis, hinge analysis and descriptive statistics. The comb area of the birds was analysed using standard curve regression analysis and correlations. The feathers counts were analysed in the same

manner as the testes. The mean of the rounded feathers on the left and right wing was used as there was no significant difference between the means, as determined through a paired *t*-test. However for the sickled feathers, the number on the right wing was used for analysis, as the number of feathers on the left and right wings were different. The semen concentration, sperm morphology and sperm motility were all analysed using standard curve regression analysis, correlations and descriptive statistics. In order to assess the sperm-egg interactions, correlations, descriptive statistics and regression analysis were performed. A binomial logistic regression was used to determine the significant effects of a number of variables on the fertility of an egg as well as to calculate the probability of attaining a fertile egg under a number of different conditions. The equation used to calculate the probability of a fertile egg (P_i) from the binomial logistic regression was:

$P_i = e^{z_i} / (1 + e^{z_i})$

where $z_i = \beta_0 + \beta_i X_i$

β_0 is the intercept

β_i is the slope

X_i is the photoperiod

3.3 RESULTS AND DISCUSSION

3.3.1 Age at sexual maturity

There was no significant effect of the final photoperiod on the ASM, as measured by the age at first semen production, of the male broiler breeders in this trial, which is unexpected due to the response of birds to constant photoperiods. Renden *et al.*, (1991) found that ASM for male broiler breeders showed a cubic response to the constant lighting treatments of 2, 4, 8, 16 or 24 h imposed from hatch to 64 weeks of age with the earliest semen production at 187 days of age (27 weeks) on 4 h of light. When comparing constant lighting treatments to changing photoperiods, Brake (1990) found an improvement in fertility for broiler breeders reared on 8 h and transferred to 10 h at 18 weeks of age when compared with the birds maintained on a constant 8 h photoperiod, which due to the lack of any significant change in female performance, was thought to be due to a possible earlier male sexual maturity. However, Brake (1990) did not determine the ASM or the

effects of the lighting treatments on the ASM of the male broiler breeders. However, the response of birds to constant lighting treatments provides evidence for juvenile photorefractoriness.

Large differences in male ASM have been reported depending on the type of chicken used. Onuora (1987) reported that sperm were first observed in the ejaculates of commercial Babcock roosters at 111 days or 15.8 weeks (no photoperiods were recorded). Aire (1973) found that Nigerian cockerels first fertilised eggs at 14 weeks of age compared with White Leghorn cockerels which first fertilised eggs at 16 weeks. Delaware roosters maintained on 1, 3, 9 and 13 h photoperiods had an average ASM of 18.6, 18.1, 17.6, and 17.1 weeks respectively (Parker & McCluskey, 1965). White Rock males raised on 6 h had a mean age at first semen production of 13.6 ± 0.3 weeks while males raised on 14 h had a mean of 12.8 ± 0.4 weeks (Siegel *et al.*, 1969) and turkey males first produced semen at 22 weeks of age when maintained on a 16 h photoperiod (Yang *et al.*, 1998).

The mean ASM for the broiler breeder males in this trial ranged from 155 ± 2.03 days of age (22.2 weeks) to 167 ± 3.01 days of age (23.9 weeks) with the males on the 9 h photoperiods maturing first while the males transferred to an 11.5 h photoperiod matured last (Table 3.3.1.1). This is similar to that of the turkey found by Yang *et al.* (1998) yet different to the ASM found in other chicken breeds. This could be due to the change in genetics, faster growth rate and heavier body weight of the broiler breeder in the last 20 years or the fact that determining the exact ASM of male birds is difficult when compared with the age at first egg in the female. It is generally accepted that the ASM of male birds is determined by the age at which a semen sample is produced for the first time through artificial ejaculation (McDaniel & Craig, 1959; Parker & McCluskey, 1965). The response of male birds to artificial stimulation and ejaculation differs between species, breed, strain and individual and may be affected by the handling environment (Lake, 1962). Ejaculation is not a spontaneous reaction to massage but rather the quantity and quality of semen will depend on the amount of training and handling the bird has experienced coupled to the degree of pressure applied to the copulatory organ (Lake, 1962). Age at sexual maturity, as measured by the age at first semen production, may not be a true reflection of the actual ASM as individual birds respond differently to training. Wilson *et al.* (1988) found that characterising males as producing or not producing semen in response to manual

semen collection was not suitable as 25% of the males that exhibited spermatogenic activity (based on testes scoring after slaughter) failed to ejaculate semen.

Table 3.3.1.1 *Mean (±SEM) age at first semen production and age at first egg for male and female broiler breeders transferred at 20 weeks from 8 h to various photoperiods between 9 and 18 h, with the difference in days between the mean age at first semen production and the mean age at first egg*

Final Photoperiod	Mean age at first semen production	Mean age at first egg*	Difference
(h)	(d)	(d)	(d)
8.0	162 ± 2.77	200 ± 2.0	38.2
9.0	155 ± 2.03	198 ± 3.0	43.0
9.5	160 ± 2.30	195 ± 2.6	34.8
10.0	164 ± 2.57	198 ± 2.5	33.8
10.5	159 ± 1.35	193 ± 2.4	34.0
11.0	160 ± 1.95	185 ± 2.2	24.2
11.5	167 ± 3.01	186 ± 2.0	18.6
12.0	160 ± 1.00	182 ± 1.4	21.5
12.5	159 ± 0.99	180 ± 1.7	20.3
13.0	160 ± 1.10	183 ± 2.3	23.3
14.0	162 ± 1.28	182 ± 2.1	19.7
18.0	160 ± 1.09	177 ± 1.1	17.1

*Data for the mean age at first egg was taken from Lewis *et al.* (2008)

Kumaran and Turner (1949a) described the growth and histological development of the testes of White Plymouth Rocks. They determined that spermatids were present in all tubules and that tubule length had increased by the 20th week of age thereby greatly increasing the capacity of the testes to produce mature sperm. De Reviers (1968) determined the duration of spermatogenesis in White Leghorns by injecting the roosters with ^{3}H-thymidine and examining the testes and ejaculates daily. It was found that spermatogenesis lasted for 6 days with the first labelled sperm released into the lumen of the seminiferous tubules 12 days after the injection and the first sperm was found in the ejaculate between days 13 and 15. Considering that the male birds in this trial first gave semen samples at 21.2 weeks of age and that the process of sperm production takes 15 days, it is possible that the birds may have been entering sexual maturity before the photoperiods had been changed at 20 weeks of age and thus the birds were already becoming sexually mature before photostimulation, resulting in no significant difference

between the ASM for different photoperiods. This statement may be supported by the results of Culbert *et al.* (1977) who found that in Thornber 909 cockerels, spermatogenesis began around 16 weeks of age when provided with 14 h of light per day, and most birds had produced semen by 24 weeks of age.

Bodyweight did not significantly affect the ASM of the males, and was not a significant variable in the multiple regression analysis, and was therefore not considered to have any effect on ASM.

Another reason for no significant effect of the final photoperiod on the mean ASM of the male broiler breeders could be the large amount of within-treatment variation (Figure 3.3.1.1) brought about by genetic and environmental sources. However, lighting did seem to have an impact on ASM in that two distinct groups could be observed (Figure 3.3.1.1) where birds on treatments ≤ 11.5 h showed a large degree of variation in ASM (149d – 180d) compared to birds on treatments ≥ 12 h (156d – 170d) who displayed a smaller degree of variation. The two groups had significantly different (p<0.001) ranges of within-treatment variations. This within-treatment variation caused the means of the treatments to be similar resulting in little difference between treatment means. The within-treatment variation was also observed in birds on the control treatment of 8 h where no photostimulation was applied (Figure 3.3.1.1). It must be remembered that the expression of phenotype is a result of genotype and environment effects and therefore this variation in phenotype could be influenced more by genetic variation than the environmental variation provided for in the different photoperiods. An increase in photoperiod to ≤ 11.5 h did not change the variation, but it was seen to decrease (even though the means stayed the same), and a more uniform response was observed, when the birds were transferred to photoperiods > 11.5 h. This could be explained by the birds responding to an increased length of light with the genetically later-maturing birds showing an advance in ASM only on photoperiods >11.5 h (Yang *et al.,* 1998; Dawson *et al.*, 2001; Lewis, 2006) while the genetically early-maturing birds could have shown a delay in the expressed ASM on photoperiods >11.5 h. However, the delay in ASM seen with the longer photoperiods when compared with the shorter photoperiods could also be attributed to handling techniques or to the length of time between sampling, as the delay was only 6 days. Assuming that the genetic variation between birds is the same on all treatments, it can said that the decrease

in variation seen in the birds on photoperiods > 11.5 h is an environmental effect and that birds on photoperiods > 11.5 h overcome some of this genetic variation and produce a more uniform phenotypic response.

The trend in ASM seen in the males is similar to that seen in the female broiler breeders subjected to the same lighting conditions (Lewis *et al.*, 2008) in that 2 distinct groups could be identified. Females transferred to ≤ 10.5 h matured to the same degree as each other and the 8 h control birds which was similar in the males except that the males on photoperiods ≤11.5 hours showed the same response. Transferring females to photoperiods ≥11 h resulted in the birds reaching maturity earlier than the 8 h controls which, in the males, was equivalent to the group on photoperiods > 11.5 h experiencing less variation in the ASM. Even though the males and females displayed similar trends, there was a significant advance in AFE of the females which was maximised by transferring the birds from 8 to 13 h, while photoperiods longer than 13 h produced no further advance in AFE (Lewis *et al.*, 2008).
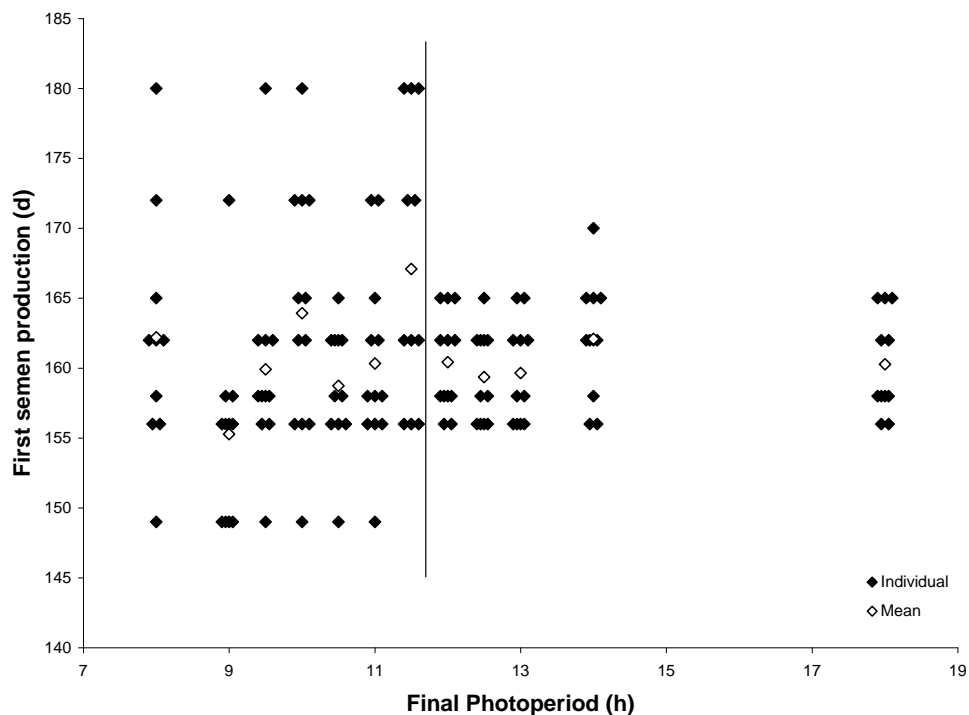
Figure 3.3.1.1 *The effect of final photoperiod on the individual and the mean broiler breeder first semen production, with the line separating two groups of birds with different degrees of variation*

Due to the male birds having no significant difference in mean ASM and the female birds having a marked significant difference in mean ASM with different photoperiods, the difference in the mean ASM (Table 3.3.1.1), measured in days, between the female and the male birds, on the same lighting treatments, can be attributed to the difference in the advance in ASM seen in the females. On the shorter photoperiods, there is a larger difference in mean ASM between the males and the females than on the longer photoperiods (Figure 3.3.1.2) which would be due to the earlier female ASM on longer photoperiods.

$$y = 0.3915x^2 - 12.771x + 120.23$$
$$R^2 = 0.82$$
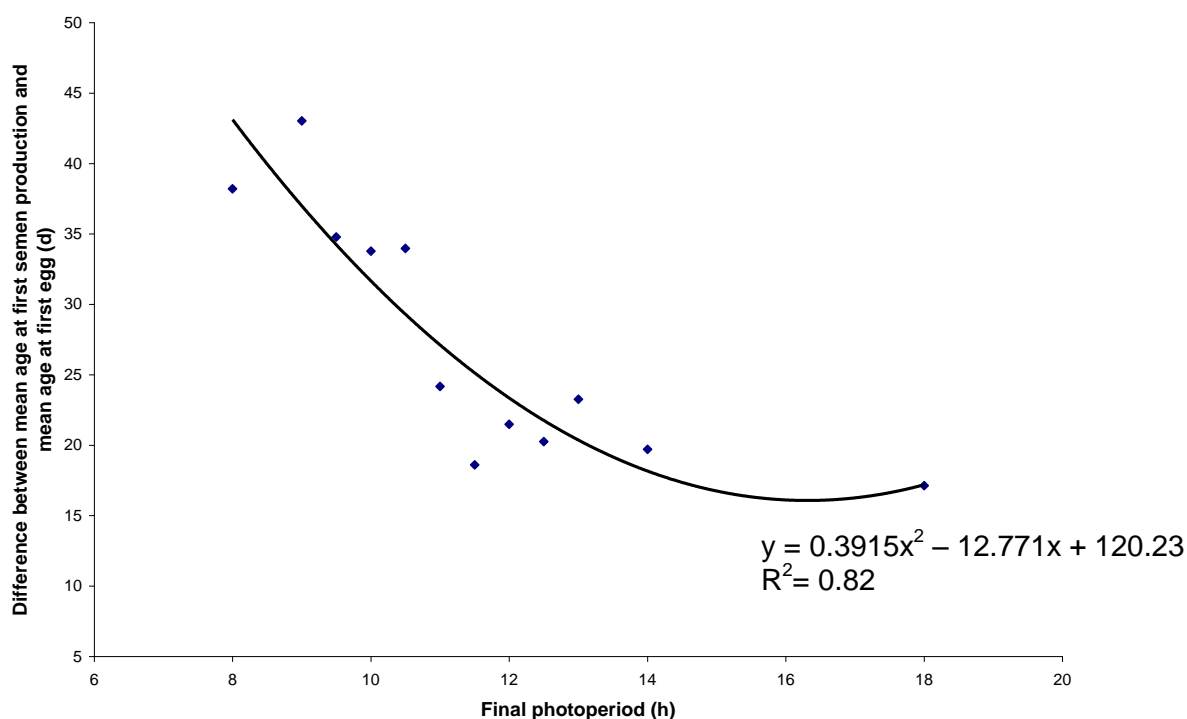
Figure 3.3.1.2 *The effect the final photoperiod has on the advance in the age at sexual maturity of male compared to female broiler breeders transferred from 8 hours of light to various other photoperiods at 144 days of age*

The mean concentration of male broiler breeder semen, measured at the corresponding female ASM was significantly affected ($p < 0.05$) by the degree of photostimulation. Two standard curves, that both had biological meaning and similar R^2 values, were fitted to explain the relationship between mean semen concentration and final photoperiod (Figure 3.3.1.3). Both curves show a decreasing trend in semen concentration at female ASM with an increase in photoperiod but Figure 3.3.1.3 b is a similar shape to that of the female advance in mean age at first egg as affected by final photoperiod (Figure 3.3.1.4). From both Figure 3.3.1.3 a and Figure 3.3.1.3 b it is evident that the male birds on the 8 h photoperiods had higher semen concentrations compared with the birds on the longer photoperiods. This is probably due to the big difference observed in male and female ASM on the same treatment (Table 3.3.1.1). As concentration of the male semen was measured at the corresponding female ASM, the male birds on the longer photoperiods had less time for the efficiency of the spermatogenic process to be at a maximum and for semen concentration to increase at female ASM because the difference in male and female ASM was less on the longer photoperiods. As seen in Figure 3.3.1.3 b, the mean semen concentration was highest for the 8 to 11 h photoperiods after which the mean concentration decreased until 13 h whereby a plateau was achieved which may indicate that the saturation daylength may have been reached. This would agree with the saturation point for female broiler breeders of 13 h that Lewis *et al.* (2008) determined. Any photoperiods > 13 h resulted in lowered semen concentration at female ASM (Figure 3.3.1.3a and 3.3.1.3b). The 8 h control photoperiod had the highest mean semen concentration at female ASM, with a value of 2304 ± 245 million sperm per ml of semen (Table 3.3.1.2). The 11.5, 12 and 14 h photoperiods displayed relatively low mean semen concentrations.
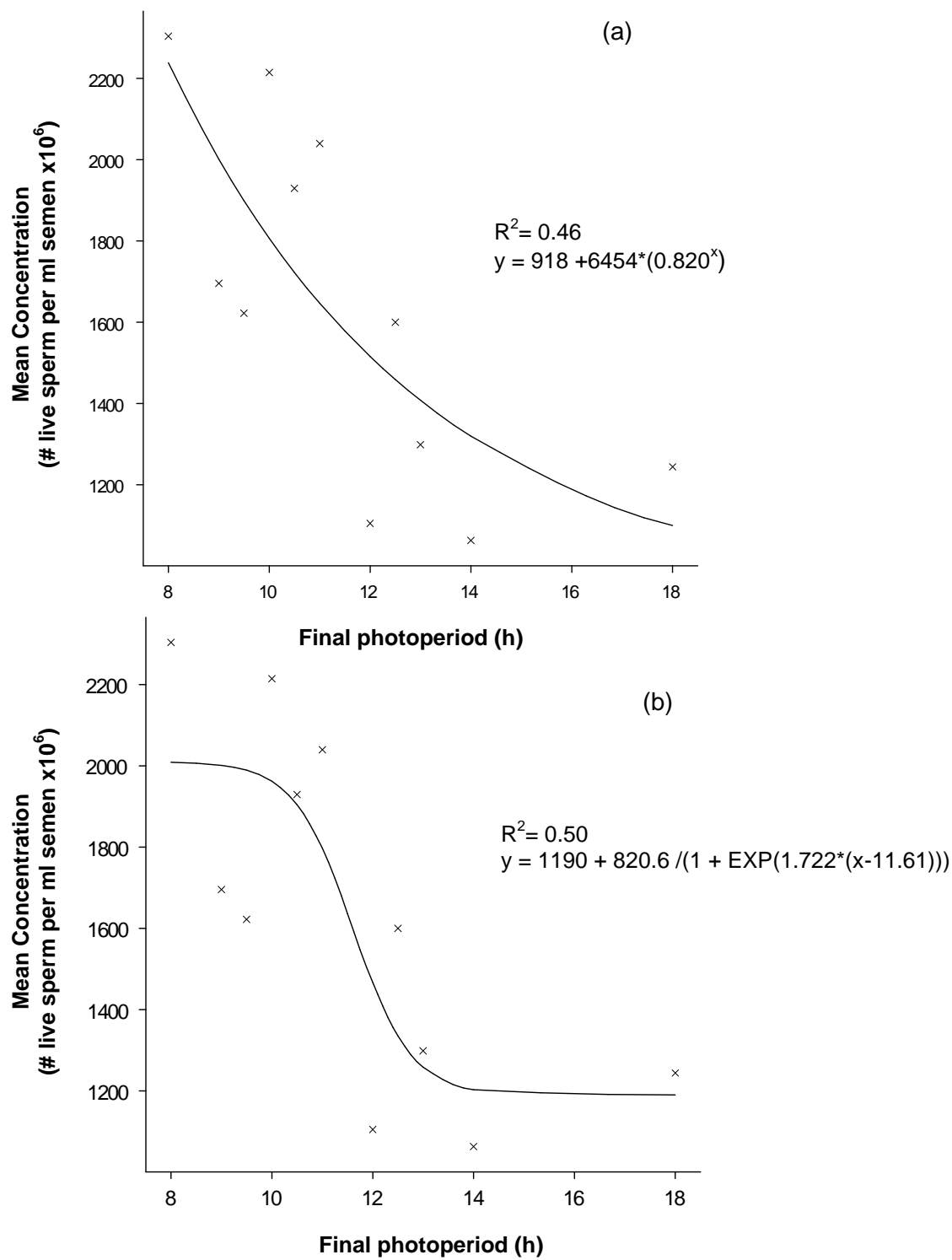
Figure 3.3.1.3 *The effect of the final photoperiod on the concentration of semen produced by male broiler breeders at female age at sexual maturity*

Since the male semen concentration in Figure 3.3.1.3b follows the same trend as the female ASM in response to photoperiod (Figure 3.3.1.4), it becomes obvious that the difference in the male and female ASM should be the main reason that the males have a low semen concentration on longer photoperiods. However, when the number of days that the male had been producing semen for when the female reached sexual maturity was considered, it was found that, despite the differences in mean semen concentration at the corresponding female ASM, where males were of different ages, there was no relationship observed from males on different treatments in mean semen concentration measured from 12 to 51d of production (Figure 3.3.1.5). Since the semen concentration of the males was not affected by the number of days that the male had been in production, it appears that the decline in the male semen concentration must be an effect of photoperiod and not the differences in ASM seen between the male and the female. Hence males transferred to photoperiods > 11 h will experience a decrease in their semen concentration and males transferred to photoperiods > 13 h will produce the lowest semen concentrations. However, there is still a large amount of variation evident between males on the same treatment indicating genetic differences that may cause individuals to respond differently on the same photoperiod. A possible explanation for this finding may be that females are generally selected for egg production whereas males are selected for meat traits which are know to be negatively correlated to fertility (Barbato, 1999). Due to a limited selection pressure for fertility or semen quality traits in males, large amounts of variation are inevitable.
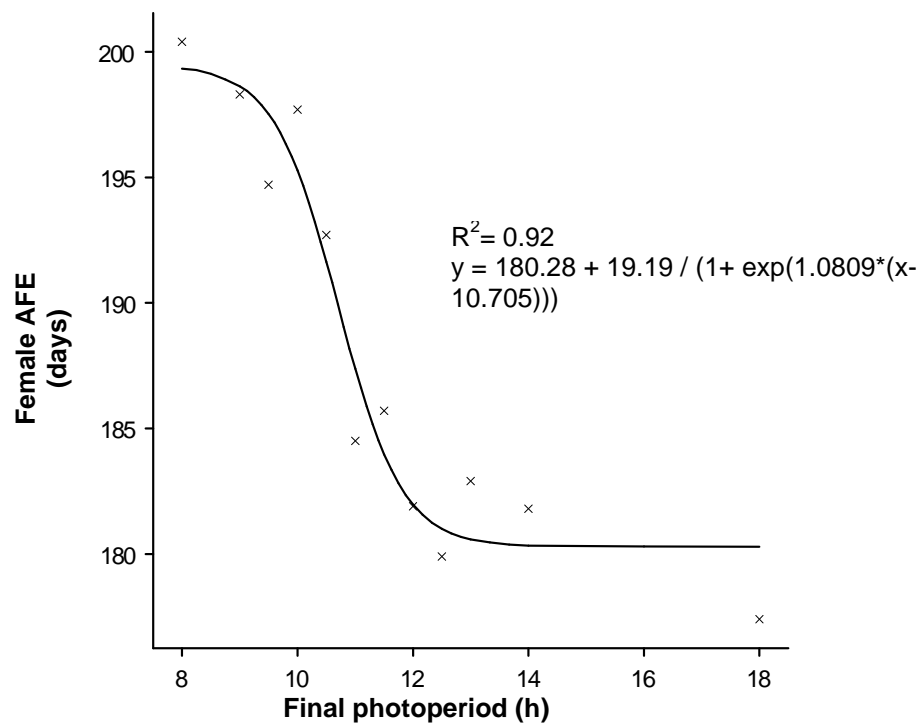
Figure 3.3.1.4 *Regression graph of the female mean age at first egg (data from Lewis et al. (2008)*
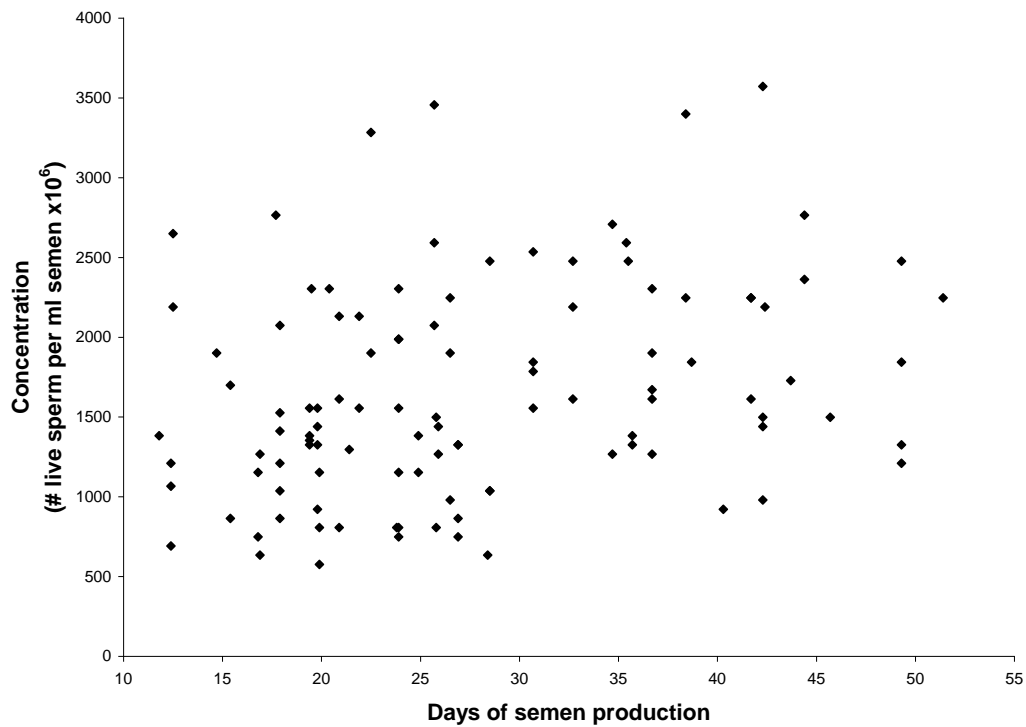
Figure 3.3.1.5 C*oncentration of broiler breeder sperm over days of semen production*

At the corresponding female ASM, there was no response of either the motility or the morphology of the male broiler breeder semen to the degree of photostimulation. These results are similar to those found by Siegel *et al.* (1969) in that neither volume per ejaculate nor sperm motility differed significantly in males raised on either 6 or 14 h of light per day. However, the results of Siegel *et al.* (1969) may indicate the effects of juvenile photorefractoriness as a result of constant daylengths. Normal sperm motility had a range of 54.5 ± 6.99 % normal sperm observed from birds on the 9 h photoperiod (which was similar to birds on the 14 h photoperiod, with a normal motility of 54.6 ± 4.74 %), to the highest value of 78.1 ± 2.49 % normal sperm from birds on the 11.5 h photoperiod (Table 3.3.1.2). The lowest percentage of morphologically normal sperm was found from birds on the 9.5 h photoperiod (22.5% ± 3.50) while the 11.5 h photoperiodic treatment resulted in the highest percentage of morphologically normal sperm (65.5% ± 4.91) (Table 3.3.1.2).

Table 3.3.1.2 *Mean (±SEM) semen concentration, % normal motility and % normal morphology for male broiler breeders transferred to various photoperiods from 8 h at 20 weeks of age, measured at the time of female ASM*

Final Photoperiod (h)	Semen Concentration (# live sperm per ml semen x10^6)	Motility (% normal)	Morphology (% normal)
8.0	2304 ± 245	69.7 ± 5.14	54.0 ± 3.82
9.0	1696 ± 282	54.5 ± 6.99	49.8 ± 1.91
9.5	1622 ± 95.0	70.0 ± 3.16	22.5 ± 3.50
10.0	2215 ± 189	64.0 ± 4.58	45.9 ± 3.00
10.5	1930 ± 142	65.6 ± 3.95	62.5 ± 2.27
11.0	2040 ± 206	75.6 ± 1.00	48.4 ± 4.37
11.5	115 ± 9.07	78.1 ± 2.49	65.5 ± 4.91
12.0	1105 ± 148	70.9 ± 3.36	61.6 ± 5.64
12.5	1600 ± 123	77.7 ± 2.18	33.6 ± 4.87
13.0	1299 ± 146	64.0 ± 4.58	47.6 ± 3.70
14.0	1064 ± 135	54.6 ± 4.74	43.0 ± 3.23
18.0	1244 ± 96.0	65.0 ± 4.23	42.4 ± 6.48

3.3.2 Testosterone

The testosterone concentration (measured at 19 weeks) did not significantly affect the ASM of the male broiler breeders in that regardless of the testosterone concentration, the

ASM was the same (22-25 weeks) across all photoperiods. The onset of spermatogenesis is associated with increased concentrations of both LH and testosterone (Sharp, 1975; Sharp *et al.*, 1977) but a significant increase in testosterone only occurs 1-4 weeks after the concentration of LH has increased (Culbert *et al.*, 1977; Sharp *et al.*, 1977). Luteinising hormone is needed to stimulate the formation of the intracellular structures responsible for steroidogensis in the Leydig cells before testosterone can be secreted (Sharp *et al.*, 1977). Testosterone is essential for the promotion of spermatogenesis as it plays a role in spermatid maturation as well as being needed for the expression of secondary sexual characteristics (McLachlan *et al.,* 1996). However, it is LH rather than testosterone which initiates sexual maturity as an increase in the mean plasma concentration of LH was found when spermatocytes had begun to form compared to an increase in the plasma testosterone concentration associated with the formation of spermatids and mature sperm (Sharp *et al.*, 1977). In this experiment, blood samples were collected at days -3, 0, +4, +7, +14 and +20 of photostimualtion for LH analysis in order to determine the exact ASM of the males but due to unforeseen circumstances, the blood was not analysed and no results for LH were obtained.

The mean plasma testosterone concentration across all treatments increased from 19 weeks to a peak around 26 weeks of age and then decreased until 30 weeks of age (Figure 3.3.2.1). These findings are in agreement with those of Sharp *et al.* (1977) who found that plasma testosterone did not increase until after 20 weeks of age, continued to rise after the onset of puberty and stabilised between 26 and 29 weeks of age in Thornber 909 roosters. In naturally mated broiler breeders, Hocking and Bernard (2000) reported that plasma testosterone increased to a peak at 30 weeks of age and then declined until a constant level was reached at 42 weeks of age. Sexton *et al.* (1989) found a linear decline in plasma testosterone concentrations from 30 to 60 weeks of age and Renden *et al.* (1991) reported an increase in plasma testosterone levels from 16 to 32 weeks of age followed by a non-significant decline in the levels to 64 weeks of age. It is possible that the shape of the testosterone curve obtained here could have changed if testosterone was measured past 30 weeks; however this was not done as testosterone was measured as an indicator of sexual maturity.
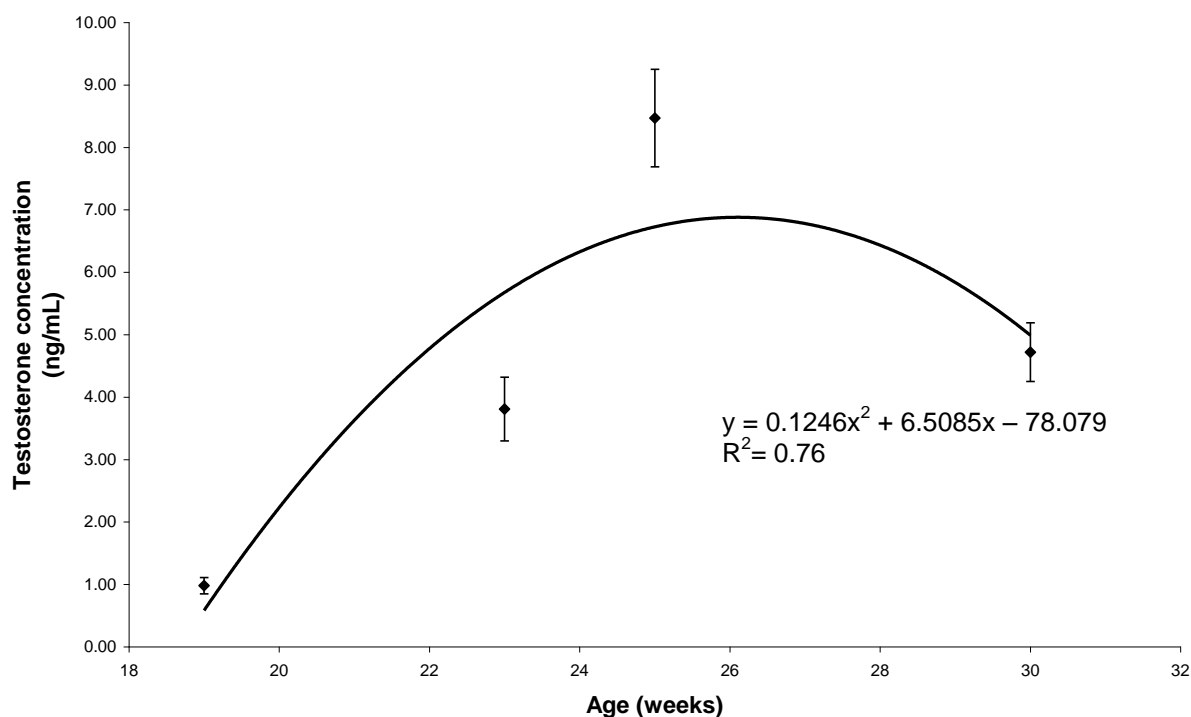
Figure 3.3.2.1 *Mean plasma testosterone concentrations (±SEM) in male broiler breeders from 19 to 30 weeks of age.*

At 19 weeks of age, the mean testosterone concentration was 0.98 ± 0.13 ng/mL indicating that, before photostimulation, testosterone concentration was low in all birds. A definite photostimulatory effect was evident from the testosterone concentration after photostimulation at 20 weeks of age and each week thereafter, the concentration was higher than the baseline of 19 weeks (Figure 3.3.2.2). At 23 weeks of age, the mean testosterone concentration was significantly affected by the final photoperiod ($p<0.05$) and tended to increase in birds on ≥10.5 h photoperiods. The within-treatment variation was at a minimum for photoperiods ≤ 11 h, above which the variation increased. The highest mean testosterone concentration was found on the 11.5 h photoperiod and the lowest on the 9.5 h photoperiod. The biggest treatment differences in mean testosterone concentration were seen at 25 weeks ($p<0.05$). Photoperiods >9.5 h resulted in increases in the testosterone concentrations. The within-treatment variation followed the same pattern as that found at 23 weeks of age. By 30 weeks of age, the testosterone concentration was not affected by the final photoperiod and the mean concentrations were

not significantly different to those at 23 weeks of age. Thus the increased photoperiod at stimulation appeared to increase testosterone concentrations at 25 weeks but by 30 weeks this effect was lost. The values of testosterone reported here agree with those found by Furr and Thomas (1970). However values found in some males in this experiment were higher, which agrees with Culbert *et al.* (1977) and Driot *et al.* (1979). The pattern of the increase and decrease of plasma testosterone levels in naturally mated birds reflected the decline in mating activity (Hocking & Bernard, 2000). Thus the testosterone levels found here are an indicator of what mating activity would have been in these caged birds considering that Sexton *et al.* (1989) and Renden *et al.* (1991) found similar testosterone concentrations in caged and naturally mated birds thereby indicating the role of testosterone in mating activity.
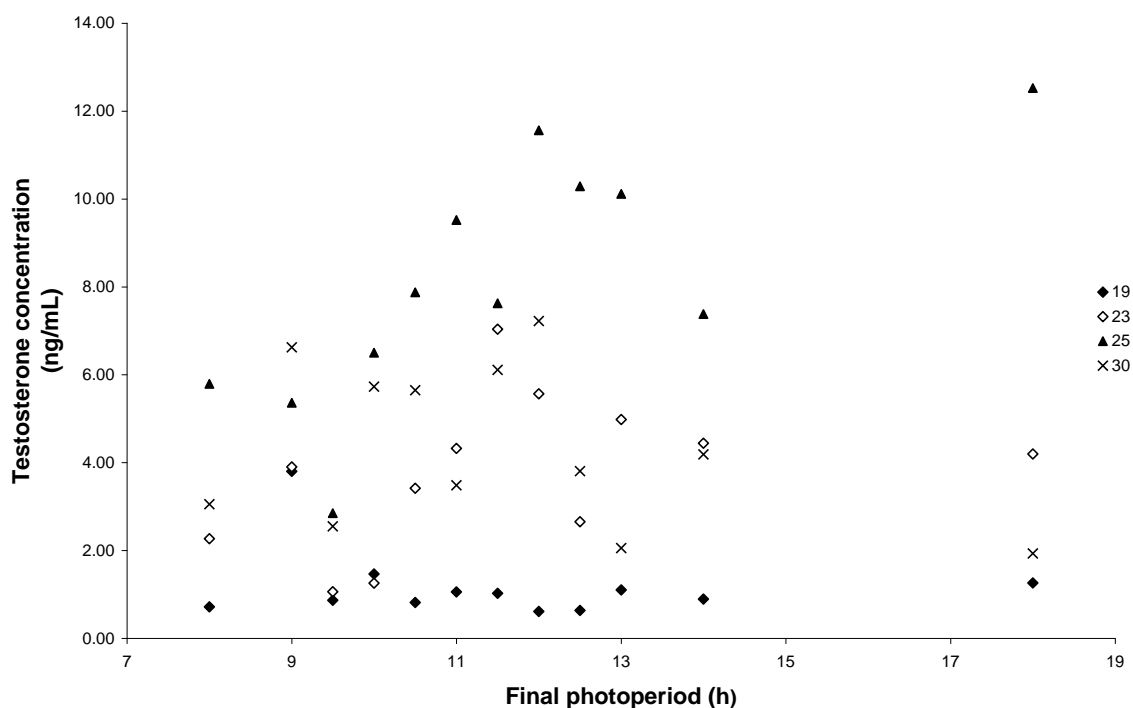
Figure 3.3.2.2 *Mean plasma testosterone concentrations in male broiler breeders from 23 to 30 weeks of age as affected by the final photoperiod (19 weeks is included as a baseline as it was before photostimulation and all the birds were on an 8 h photoperiod)*

When male Japanese quail were exposed to various photoperiods ranging from 12 to 20 h, it was found that plasma testosterone levels were affected by the photoperiod, with lower levels of testosterone found in birds on < 12 h than in birds on 20 h after only 4 days of photostimulation (Follett & Maung, 1978). In male turkeys, Yang *et al.* (1998) found that plasma testosterone levels were influenced by photoperiod, with higher testosterone levels found on the long-day photoperiods (birds maintained on 16 h light throughout the trial) when two groups of birds were maintained on either 6 h of light from 12 to 29 weeks of age and then transferred to 16 h until 35 weeks or on 16 h of light from 10 to 35 weeks of age. After transfer of the males on the short-daylength to the long daylength (16 h), the testosterone levels increased significantly within days of the change in photoperiod. Kurginshi Noonan and Bacon (2000) found that early lighting of male turkeys resulted in more stable levels of plasma testosterone than the rearing of males on traditional lighting programmes. In broilers, plasma testosterone levels were significantly affected by the lighting schedule imposed, in that males on an intermittent lighting schedule had elevated plasma testosterone concentrations when compared with males kept on the constant control photoperiod of 23 h light (Kuhn *et al.*, 1996). Renden *et al.* (1991) reported that a positive relationship exists between the plasma testosterone levels and the hours of light provided in broiler breeder males. The findings of these authors support the results of this trial, such that a photoperiodic effect was evident on the testosterone concentrations (Figure 3.3.2.2) of the male broiler breeders after photostimulation at 20 weeks of age. The high amount of variation in the individual testosterone concentrations found on the different photoperiods may be caused by the genetic makeup of the individual birds as was found by Sharp (1975) with the mean concentration of LH in the plasma of growing cockerels varying greatly between individuals (Sharp *et al.*, 1977).

Testosterone, in this study, had a significant effect on comb area ($p < 0.001$). The comb area increased with an increase in testosterone concentration until a plateau was reached after which comb area did not increase regardless of the testosterone concentration (Figure 3.3.2.3).
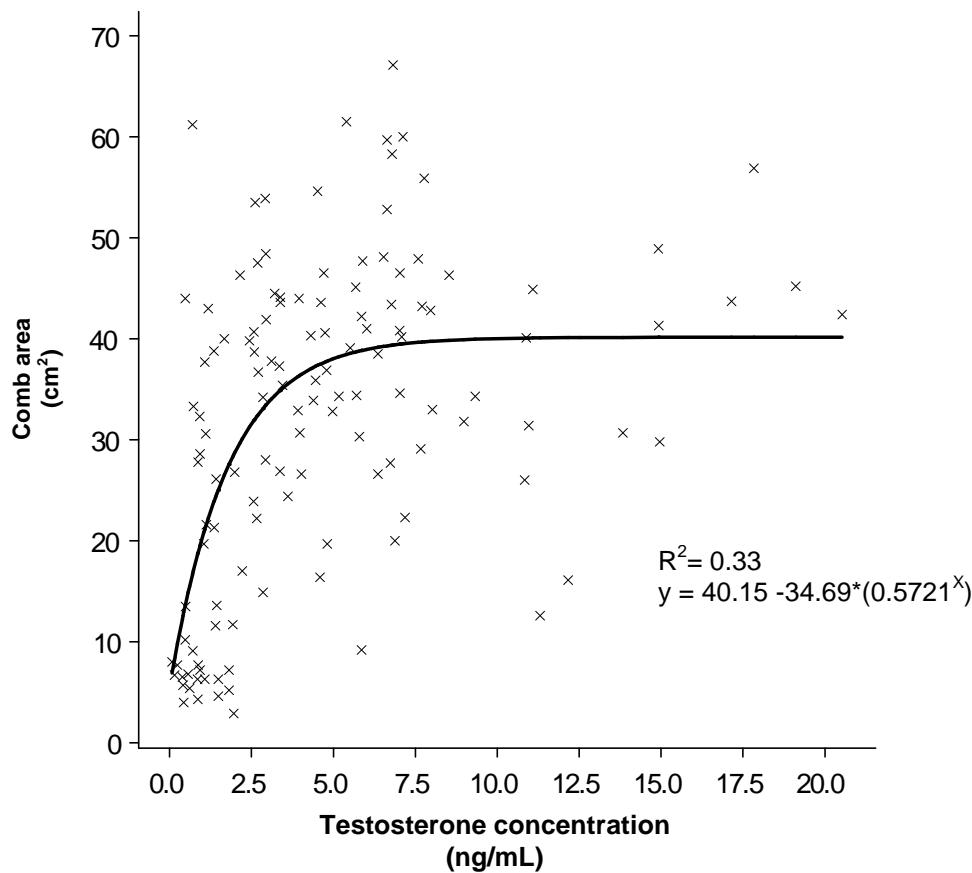
Figure 3.3.2.3 *The effect of testosterone concentration on comb area*

Plasma testosterone concentration was found to be positively correlated with age (up to 30 weeks of age) (r= 0.56). Sperm motility was significantly correlated with testosterone concentration (r= 0.45) at 30 weeks of age but not at 25 weeks. This may suggest that a strong relationship between testosterone and motility is only initiated or seen when testosterone levels start to decline. Neither semen concentration nor sperm morphology was significantly correlated with the testosterone concentration at either 25 or 30 weeks of age.

3.3.3 Secondary sexual characteristics

At the age at which female broiler breeders, reared under the same conditions as the males in this trial, reached sexual maturity, there was no significant photoperiod effect on the predicted testis weights of the males. It is generally accepted that males mature earlier

than their female counterparts in order for the males to have reached a high level of sexual activity before the females start to ovulate (Raitt & Ohmart, 1966; Rosa & Bryant, 2003). In order for this to happen, the male reproductive system has to have matured in the areas of hormone production, testis growth and spermatogenesis. Thus, it would be expected that the testes of the male would be mature by the age at which females attained sexual maturity and so would not be affected by the final photoperiod at that age.

The results of t-tests between the predicted testis weight and the actual testis weight at slaughter showed that the prediction equation developed by Tyler and Gous (2006) for younger birds could not be used to accurately predict the testis weights of male broiler breeders at 51 weeks of age.

At 51 weeks of age, the left testis weighed more than the right testis for 67.7% of the males. This result concurs with that found by Hocking (1992) where after examination of the testes from 378 males, the left testis was found to have a greater weight than the right testis in 66.9% of the males. The results of t-tests between the mean of the left and right testis weights showed that the left and right were not significantly different for birds from the majority of the photoperiods except for 11.5, 12, 12.5 and 14 h which are the middle range of photoperiods excluding 13 h. This is in agreement with Hocking (1992) where, in general, the left and right testes were not significantly different in weight.

As can be seen in Figure 3.3.3.1, the right testis regressed at a faster rate than the left and the left testis weighed more than the right at 51 weeks. Three distinct groups can be seen in Figure 3.3.3.1, the first group in which testes weights were high and regression had not yet started (8-10 h), the second group where the weights were average and were decreasing at a fast rate indicating the rate of regression (>10-12.5 h) and the third group where the weights were low showing that the testes had probably already regressed in size (>12.5 h). It is well known that long photoperiods result in the induction of photorefractoriness (Nicholls *et al.*, 1988; Dawson *et al.*, 2001) and this is probably the reason that the males on the longer photoperiods had testes of a smaller size as gonadal regression occurs during photorefractoriness.
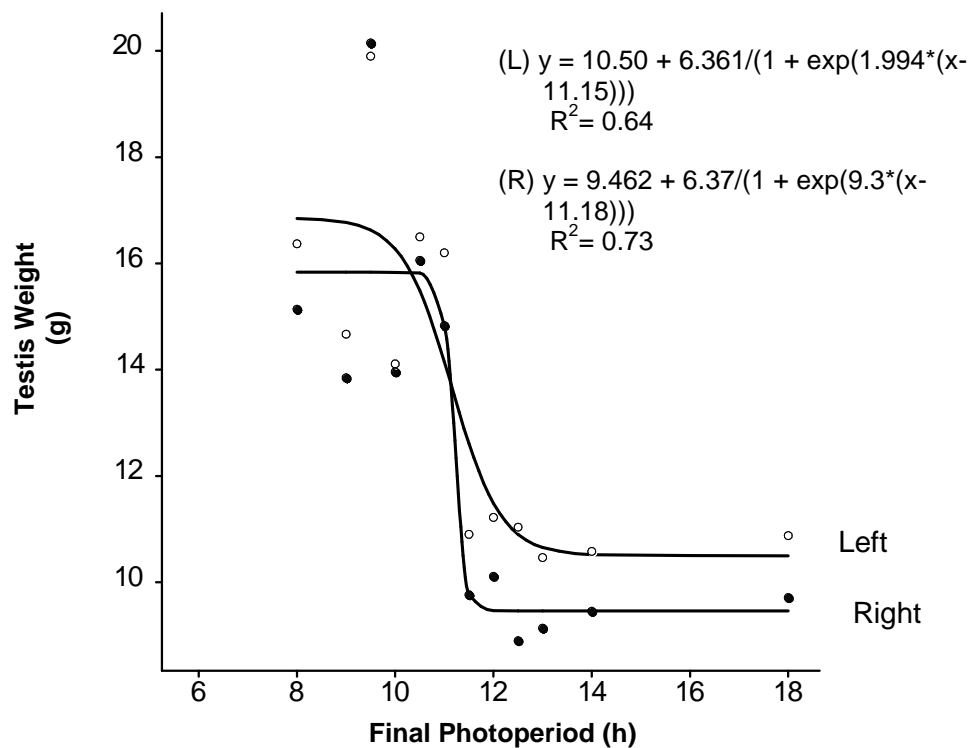
Figure 3.3.3.1 *The mean testis weights for the left and right testes as affected by the final photoperiod at 51 weeks of age slaughter*

At 51 weeks of age, the final photoperiod did have a significant effect on the average testes weights of the male broiler breeders and a hinge point was found at 13 h (Figure 3.3.3.2). Thus males on final photoperiods >13 h had average testes weights lower than males on photoperiods ≤ 13 h (Figure 3.3.3.2). On average, the mean testes weights for photoperiods ≥ 11.5 h did not exceed 11g and photoperiods ≤ 11 h were no lower than 14g. This provides further evidence of the early onset of adult photorefractoriness in birds on longer photoperiods. Based on these results, the evidence of testes regression upon slaughter (Figure 3.3.3.1) and the literature, it would seem appropriate to assume that the onset of adult photorefractoriness in birds on the longer photoperiods had begun.
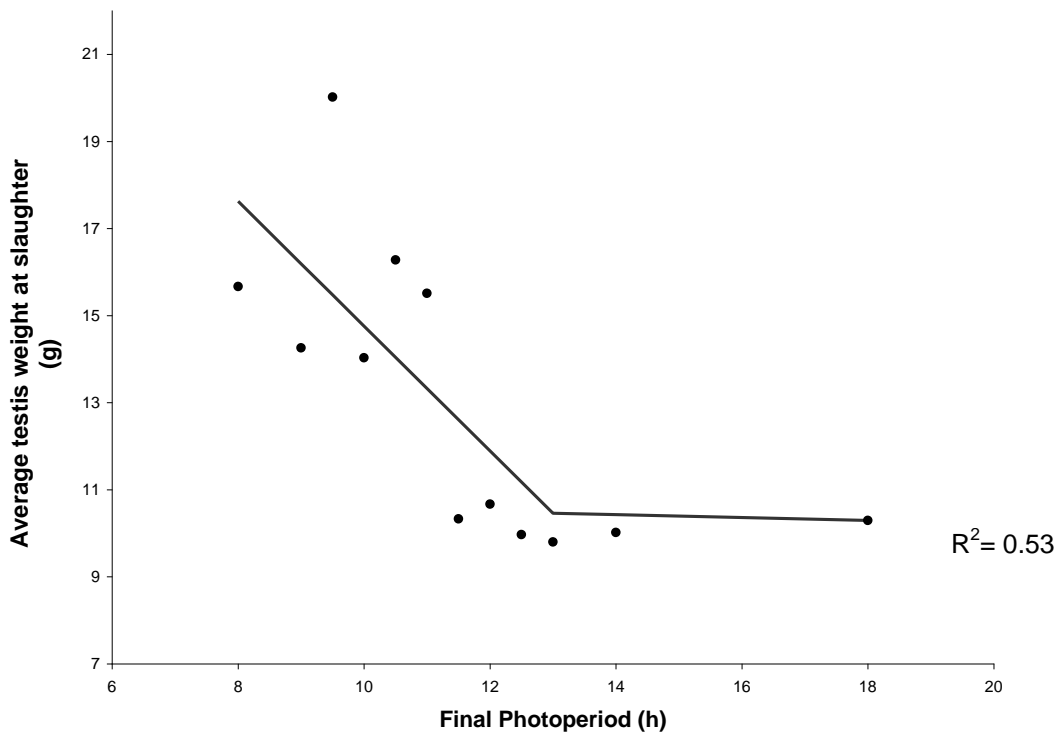
Figure 3.3.3.2 *Hinge regression analysis of mean testes weights on final photoperiods for broiler breeder males at the time of slaughter*

By 51 weeks of age, there was also some variation observed from males on the same treatments (Table 3.3.3.1), where some males within a treatment had undergone testes regression and were no longer producing semen yet others showed no signs of impending testes regression (Figure 3.3.3.3). This variation was similar to that seen at male ASM (Figure 3.3.1.1) and implies that male birds seem to have an individual response to light. However, the practical implication for this response is that the treatment averages are high therefore the males which have larger testes would compensate for the males in the flock which have smaller testes. All males which had no semen in the vas deferens were on photoperiods ≤ 12.5 h except for one male on the 14 h photoperiod. The highest average testes weight was found on the 9.5 h photoperiod and the lowest on the 12 h photoperiod (Table 3.3.3.1).

(a)   (b)

Figure 3.3.3.3 *Photographs showing the difference in size and vascularisation of testes from two different male broiler breeders on the same lighting treatment (14 h) upon slaughter at 51 weeks of age*

Table 3.3.3.1 *The treatment average (± SEM) of average left and right testis weights, and the highest and lowest average of left and right testes weights of male broiler breeders on each photoperiod at time of slaughter (51 weeks)*

Final Photoperiod	Treatment Average of average left and right testes weights	Highest average left and right testis weight	Lowest average left and right testis weight
(h)	(g)	(g)	(g)
8.0	15.7 ± 1.99	22.0	9.84
9.0	14.2 ± 0.53	16.7	13.00
9.5	20.0 ± 1.88	25.8	16.10
10.0	14.0 ± 1.23	16.4	11.40
10.5	16.3 ± 0.38	18.2	15.30
11.0	15.5 ± 0.83	18.5	12.20
11.5	10.3 ± 1.60	14.0	5.61
12.0	10.7 ± 2.45	17.9	2.56
12.5	10.0 ± 2.79	17.0	3.35
13.0	9.8 ± 1.66	12.7	6.17
14.0	10.0 ± 2.66	14.0	2.26
18.0	10.3 ± 1.80	14.5	4.89

It was noted upon slaughter, that no bird with an average testis weight < 10g produced semen (Table 3.3.3.2), except for two birds on the 18 h treatment in which semen was observed in the vas deferens even though the average testis weights were 5.19 and 4.89g. Of these two birds, the semen sample collected from the one was contaminated and thus not analysed for semen concentration, sperm motility and morphology but the other produced a sample which was analysed. It was found that this bird produced the lowest semen concentration, normal motility and normal morphology out of all the birds analysed for that treatment. This is further confirmation of the fact that it is better to have a high average flock fertility and bigger testes with these males compensating for some poor producers rather than having a low fertility with many birds producing poor semen as a result of low testes weights. Vizcarra *et al.* (2004) reported that in male broiler breeders a normal (producing semen) testis size was > 10g. Sharp *et al*. (1977) found that only when the testes (average of left and right testis) of birds weighed more than 10g was spermatogenesis completed and a significant rise in the mean plasma concentration of testosterone observed. The findings in this experiment are in agreement with those of Sharp *et al*. (1977). By estimating the point at which the birds stopped producing semen it became apparent that the majority of birds with testes weights <10g had stopped producing semen by 45 weeks of age with the exception of two males; one which had stopped producing within the 51st week (between the collection of the sample at the beginning of the week and slaughter at the end of the week) and the other which had stopped production at 25 weeks of age. This appears to be consistent with the typical fertility trend of commercial broiler breeder flocks where fertility tends to increase to a peak at 30 to 40 weeks of age and subsequently declines (Hocking & Bernard, 2000).

Table 3.3.3.2 *Time at which male birds lacking semen in the vas deference at slaughter probably stopped producing semen and the mean testis weights at slaughter*

Final Photoperiod (h)	Cage Number	Week 33	Week 45	Week 51	Mean testis weight at slaughter (g)
8.0	8	✓	✓	X	9.84
9.0	1	✓	✓	X	6.17
11.5	2	X	X	X	7.71
11.5	5	✓	X	X	7.53
11.5	9	✓	✓	✓	5.61
12.0	4	✓	X	X	2.56
12.0	11	✓	X	X	5.01
12.5	4	✓	X	X	4.43
12.5	9	✓	X	X	3.35
14.0	2	✓	X	X	2.26

✓ producing semen   X not producing semen

Considering that there was a large amount of variation surrounding the average testis weights of the male birds at slaughter (Table 3.3.3.1), a limited number of birds with regressed testes (< 10g), no final photoperiodic effect on the regressed testes (< 10g) and two males with testes < 10g still producing semen, it may be assumed that broiler breeder males either undergo testis regression at an age later than 51 weeks or that the testes of the male regress in size but do not stop producing semen but rather decrease the volume or concentration of semen produced. As the volume of semen produced was not recorded, it is not possible to say whether the volume of semen decreased or not. This is the case in male sheep that are known to be seasonal breeders as testis weight can decrease along with sperm production but spermatogenesis and sexual activity never actually stop (Rosa & Bryant, 2003). Genetics may play a major role in the reproductive responses to light and testis regression of male birds (as seen by the large amount of variation) and in the future it may be worth selecting roosters which display the onset of adult photorefractoriness at an older age or which do not stop producing semen altogether.

There was no significant difference between the widths and the lengths of the left and right testes, however of the 65 birds slaughtered at 51 weeks of age, 63.1% of the birds had a left testis wider than the right and 83.1% of the birds had a left testis longer than the right which probably accounted for the weight in 67.7% of the birds.

No relationship was found between the average testis weights and any aspect of semen quality measured. It is well known that generally the ejaculate volume increases with an increased testes weight and/or size (Amann, 1999; Møller, 2008). However, in this trial volume was not measured as it was thought that the semen quality measures of concentration, motility and morphology were more indicative of the fertilising ability of the male than the volume of semen collected. It was also felt that differences in volume may have arisen while trying to prevent faecal contamination which may have skewed results.

The comb area of the broiler breeder males increased with an advance in age and was found to be highly correlated with age (r = 0.6471). A fast rate of growth of the comb area was seen from 19 to 21 weeks of age after which the growth rate tended to decrease but the area of the comb continued to increase until the birds were slaughtered at 51 weeks of age (Figure 3.3.3.4). Sharp (1975) found that in pullets, like in cockerels, the accelerated rate of growth of the comb indicated the imminent onset of sexual maturation at 16 to 19 weeks of age and that the comb ceased to grow rapidly around the time that the first eggs were laid at 23 to 30 weeks of age. Similar results were found by Sharp *et al.* (1977) as a significant (p<0.05) increase in the size of the comb was seen between 16 and 24 weeks of age and that comb growth continued after the onset of puberty and stabilised around 26 weeks of age.
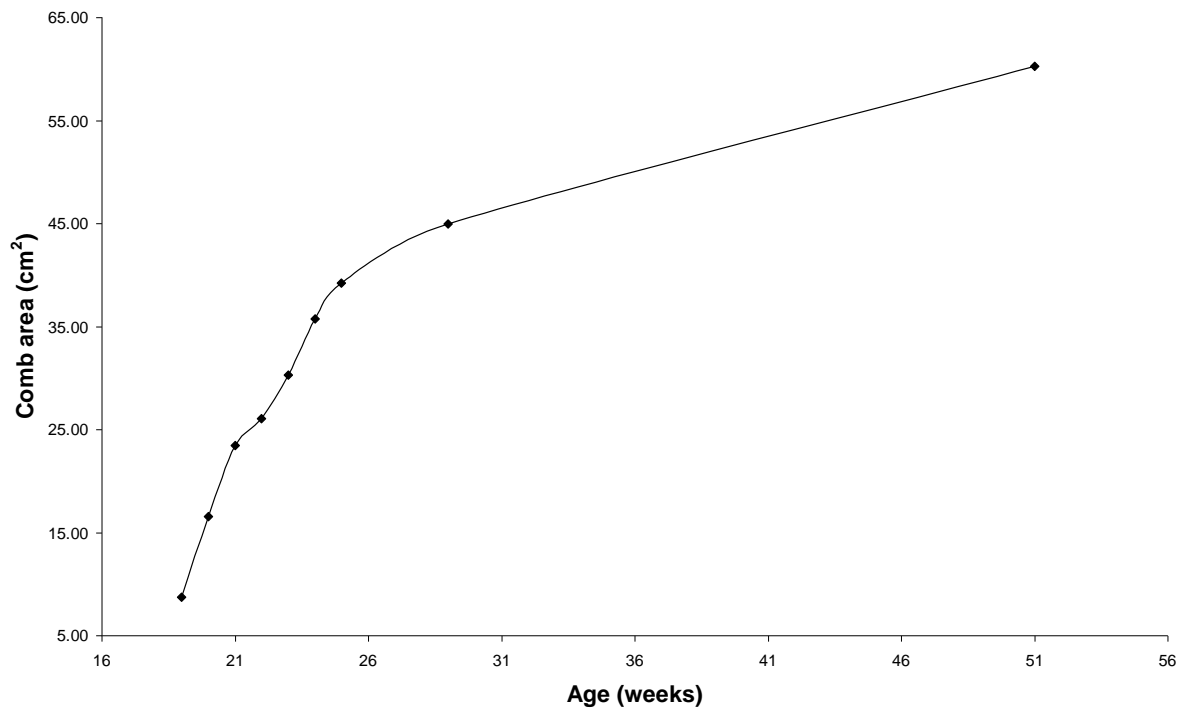
Figure 3.3.3.4 *The change in comb area with age*

Generally the area of the comb was not affected by the final photoperiod, however, a significant effect ($p<0.05$) of photoperiod on comb area was observed at weeks 29 and 51. Parker and McClusky (1964 and 1965) ran two experiments in which conflicting results regarding the effect of light on comb area were determined. In the first trial (1964), the comb size was not related to the amount of daily light when the males were given 1, 3, 9 or 13 h of daily light starting from 285 days of age (males were reared on differing light schedules similar to the treatments in that 3 of the males received the same amount of light given them in the experiment, 3 had received one of the other lighting treatments and 1 was exposed to natural light). In the following experiment (1965), where males were given 1, 3 and 9 h of light from hatch, the comb size increased with increased amounts of light. The male birds maintained on 13 h of light from hatch had the smallest comb size at 16 to 20 weeks of age when compared with the males maintained on 1, 3 or 9 h and the authors concluded that comb size was not always related to the length of the photoperiod provided. However, the issue of juvenile photorefractoriness arises as constant photoperiods may take longer to dissipate this.

When the comb area and the semen characteristics of concentration, sperm motility and morphology were considered, no significant correlations were found. These results were in agreement with those found by Burrows and Titus (1939), Pizzari *et al.* (2004) and Bilcik *et al.* (2005). This was to be expected considering that comb area is a testosterone-mediated trait and low or negative correlations were found between testosterone and semen characteristics.

At 51 weeks of age, it was found that the average testis weight was correlated with the area of the comb (r = 0.4956) over all photoperiods. This result corresponds with those found by other researchers such as Breneman (1937) who detected a definite correlation between comb size and gonad weight and McGary *et al.* (2002) who reported a significant positive correlation (r = 0.4735) between comb area and testes weight.

At slaughter, some of the male birds who had average testis weights < 10g and were not producing semen, had also undergone a regression in the area of the comb when compared with areas measured at 29 weeks of age (Figure 3.3.3.5). Of the ten birds not producing semen at slaughter, 4 had undergone comb area regression, 2 had maintained the same area (or possibly grown and regressed between 29 and 51 weeks of age) and 4 had increased in area. This phenomenon of comb regression was demonstrated by Hill and Parkes (1934) who found that the comb, wattles and testes of roosters regressed after hypophysectomy and concluded that this result was similar to that found after both castration and moult. A possible explanation is that testis regression, mediated through apoptosis, caused fewer Leydig cells to be present or those present to be less responsive and therefore less testosterone was produced (Young *et al.*, 2000) to maintain the size of the comb, since comb size is known to be testosterone dependent. Vizcarra *et al.* (2004) described the gonadotropin secretion in male broiler breeders and indicted that in a male with a small testis size (< 10g) the Leydig cells did not respond to LH impulses in the same manner as they did in birds with normal sized testes (> 10g) and, thus, resulted in a reduced testosterone pulse frequency, amplitude and baseline. In male grouse, Mougeot *et al.* (2005) found that comb size was influenced by testosterone and when testosterone concentrations declined during the non-breeding season, so did comb size. Considering that testosterone concentrations were not determined at this stage of the trial (51 weeks),

it is impossible to say if the regression in comb size seen here is testosterone-mediated or not. During the breeding season, it is well known that males with large combs have lower levels of lymphocytes but a greater cell-mediated immunity. However at other times of the year, the lymphocyte proportion increases (Zuk & Johnsen, 1998; Mougeot *et al.*, 2004). A possible hypothesis regarding the regression of the comb may be that of the comb regressing in size during the non-breeding season in order to allow for the proportion of lymphocytes to increase, thereby ensuring that there are enough lymphocytes available for the breeding season. However, no literature could be found to support this hypothesis as all the literature regarding comb regression suggests that it is testosterone mediated.

(a)   (b)

Figure 3.3.3.5 *The change in comb area from 49.5cm^2 at 29 weeks of age (a) to 19.1cm^2 at 51 (b) weeks of age of an individual bird on a final photoperiod of 11.5 h*

In seasonal breeding birds, reproduction and moult do not occur simultaneously but rather moult begins immediately after breeding (North, 1972; Ruszler, 1998; Dawson *et al.*, 2001). There are two mechanisms by which moult is induced which include the photoperiodic effect of long days and the physiological relationship (yet unknown) between the end of breeding and the beginning of moult such that the start of moult is delayed if breeding continues past the photoperiodic cue for moult (Dawson *et al.*, 2001). At 51 weeks of age, it appears that the male broiler breeders had not yet begun to moult as the majority of them were still producing semen and only four had lost the greater part of their primary wing feathers. Of these four birds, one had stopped producing semen and had

undergone testes regression but the remaining three were still producing semen. It could be possible that, at this age, moult was only starting to begin as there was a moderate negative correlation found between age and the total number of primary feathers on the wing (r=-0.214) but a significant negative correlation was found between the sickle feathers and age (r=-0.4733). No significant photoperiodic effect on the number of primary feathers, sickle or rounded, was evident. Raitt and Ohmart (1966) reported that most male quails in the early stages of moult possessed regressing testes. The authors followed on to say that there was a large amount of variation regarding this statement as some males well into the moult had active testes or testes in very early regressive stages and a few birds which had fully regressed testes had not begun to moult. They concluded that the onset of both regression of the testes and moult occurred around the same time but were both totally independent processes and it was doubtful if they were related or causative of each other. When the testes and feathers of the broiler breeder males in this experiment were examined at slaughter it was found that a small relationship existed between the two in that average testis size and the number of sickle feathers were positively correlated (r=0.166) and the number of rounded feathers was negatively correlated with the average testis weight (r=-0.236). No significant relationship was found between the total number of primary feathers and average testes weights ≤ 10g. This suggests that when testis regression occurs, the rounded feathers replaced the sickle feathers from the axial feather to the tip of the wing, in the usual order of moult, when the testes start to recrudesce. This was evident by the high correlation found between the number of sickle and rounded feathers (r=-0.830) and is illustrated in Figure 3.3.3.6. However, the loss and replacement of feathers may not have been due to moult but merely instigated by the damage of the feathers caused by the birds flapping their wings against the cages and then having to replace them. As the order of the feather loss and replacement and the age at which the first primary feather was lost were not recorded, it was impossible to determine if the loss and replacement of the feathers was due to moult or cage damage. Considering that low-producing birds moult slowly, one primary feather at a time, compared with high-producing birds who may moult up to four primary feathers at a time (North, 1972), this slow moult might mean that the replacement of the feathers as indicted by the number of primaries may not have been noticed and thus underestimated.
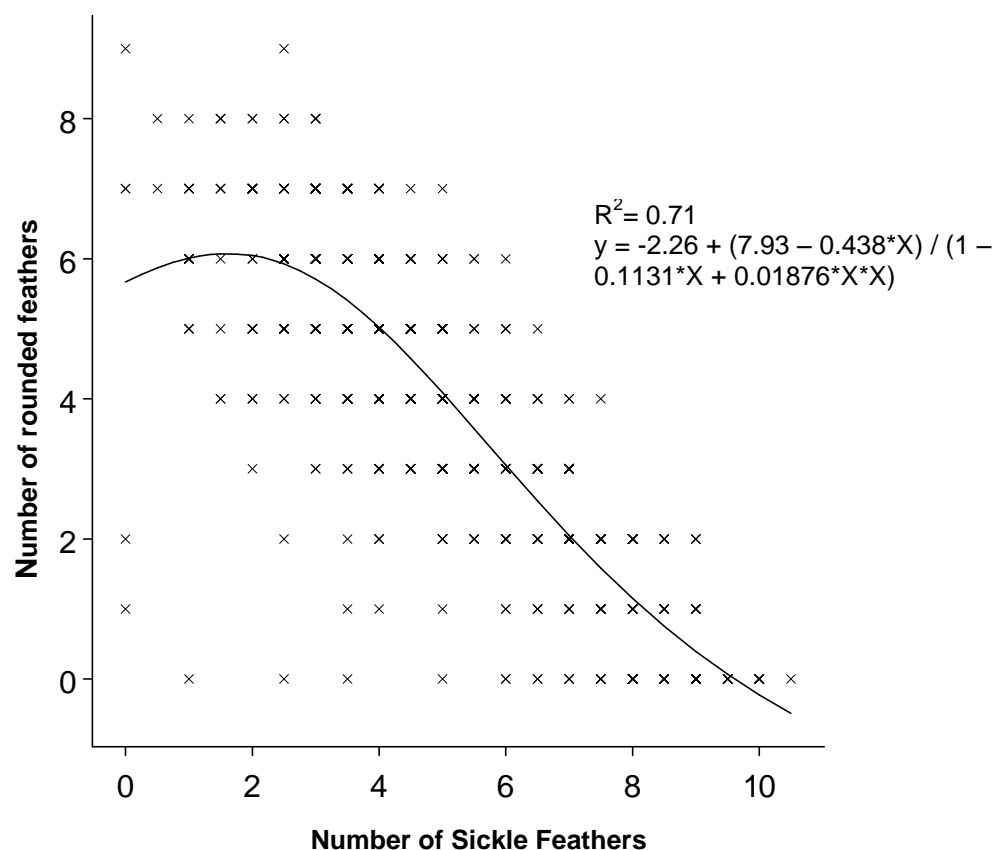
Figure 3.3.3.6 *The relationship between the primary wing feather types of caged male broiler breeders*

3.3.4 Semen analysis

The final photoperiod did not significantly affect any of the semen characteristics (semen concentration, sperm motility or sperm morphology) of the male broiler breeders at 33, 45 or 51 weeks of age. In the literature this seems to be a controversial area as some authors have found that the photoperiod does affect certain semen characteristics (Proudfoot, 1981; Renden *et al.*, 1991) yet others disagree (Parker & McSpadden, 1943; Bajpai, 1963; Siegel *et al.*, 1969). Most of the tests that are used to determine these characteristics are highly subjective and sometimes are based on a scoring technique which leads to the results often being unrepeatable and differing between researchers. This problem lead to the development of computer aided semen analysis techniques, however, these are not popular due to the high cost. The ability of these measures of semen characteristics to

accurately predict a male's fertility or fertilising ability is still controversial and has led to the development of more reliable tests of fertilising ability such as the sperm-egg binding assay. Further, the problem of subjectivity is confounded by the large amount of variation seen within photoperiodic treatments, individuals and their semen characteristics. This variation could be due to the male birds not being selected for sexual traits but rather for those of carcass quality and yield.

The lowest mean semen concentration was found at 33 weeks of age (835 ± 76.2 million sperm per ml of semen on 18 h) and the highest mean concentration at 51 weeks of age (2659 ± 273 million sperm per ml of semen on 8 h) (Table 3.3.4.1). The birds on the 8 h photoperiod tended to have the highest semen concentration when compared with the other photoperiods, with values of 1958 ± 201, 2858 ± 418 and 2659 ± 273 million sperm per ml of semen for 33, 45 and 51 weeks of age.

However, a large amount of variation was evident within a treatment. At 33 weeks of age, for example, the birds on the 14 h photoperiod produced semen concentrations which ranged from 922 to 3283 million sperm per ml of semen. The highest semen concentration (3283 million sperm per ml of semen) was obtained from birds on the 14 h treatment and the lowest (576 million sperm per ml of semen) on both the 12 and 13 h photoperiods. By 45 weeks of age, the variation was less where the highest semen concentration had decreased and the lowest had increased when compared to 33 weeks of age and were calculated as 3197 (11.5 h) and 893 (8 and 13 h) million sperm per ml of semen respectively. The birds maintained on 11.5 h were all high producers when compared to the birds maintained on the other photoperiods as could be seen by a mean of 2442 million sperm per ml of semen. The lowest semen concentration measured for the 11.5 h photoperiod was 1843 million sperm per ml of semen and thus variation within the treatment, even for these, on average, high producers was evident. By 51 weeks of age, the variation had again increased with the highest semen concentration increased to 4838 million sperm per ml of semen (10.5 h) and the lowest concentration decreased to 835 million sperm per ml of semen (14 and 18 h). The high variation and no photoperiodic response suggest that there are other factors controlling semen concentration.

Table 3.3.4.1 *Mean (±SEM) semen concentration of male broiler breeders transferred from 8 h to various other photoperiods at 33, 45 and 51 weeks*

Final Photoperiod	Concentration		
(h)	(# live sperm per ml semen x10^6)		
	Week 33	**Week 45**	**Week 51**
8.0	1958 ± 201	1454 ± 174	2659 ± 273
9.0	1066 ± 177	1501 ± 188	1660 ± 119
9.5	1521 ± 153	1872 ± 180	1622 ± 123
10.0	1015 ± 176	1559 ± 155	2006 ± 205
10.5	1620 ± 81.0	1958 ± 108	2376 ± 387
11.0	922 ± 127	1926 ± 175	1776 ± 186
11.5	1709 ± 122	2442 ± 234	2237 ± 167
12.0	1375 ± 289	1550 ± 189	1798 ± 263
12.5	1472 ± 84.0	1619 ± 115	1542 ± 77
13.0	1210 ± 156	1454 ± 174	1904 ± 134
14.0	1692 ± 271	1624 ± 227	1910 ± 307
18.0	835 ± 76.2	1872 ± 180	1558 ± 161

The mean normal motility tended to increase from 33 to 51 weeks of age for all the treatments except 10 h where the mean motility stayed the same (Table 3.3.4.2). The mean normal motility for the 18 h photoperiod at 33 weeks of age was very low at 25 % but this could be attributed to the mean being calculated from only 2 samples out of 12 birds on the treatment for that day of collection. The highest mean percentage of normally motile sperm was 84.4 ± 2.69 % on 10.5 h of light at 45 weeks of age. Besides the very low value of 25.0 ± 0.00 % (all the measurements were the same) motile for 18 h at 33 weeks of age, the second lowest value was 55.6 ± 3.95 % for 13 h at 33 weeks of age. Motility of individual birds displayed a large amount of variation within a treatment; however this variation tended to lessen over the weeks. An example of the large variation was seen in the 10.5 h photoperiod in which individual male motility ranged from 40 to 90%. As with semen concentration, motility measurements did not show a photoperiodic response.

Table 3.3.4.2 *Mean (±SEM) sperm motility of male broiler breeders transferred from 8 h to various other photoperiods over time*

Final Photoperiod	Motility		
(h)	(% normal)		
	Week 33	**Week 45**	**Week 51**
8.0	65.0 ± 5.00	79.5 ± 3.76	80.6 ± 1.76
9.0	70.0 ± 5.70	75.6 ± 4.77	68.1 ± 2.66
9.5	65.5 ± 3.37	71.7 ± 4.49	80.8 ± 3.52
10.0	65.0 ± 3.93	65.0 ± 6.45	65.0 ± 4.17
10.5	67.5 ± 5.26	84.4 ± 2.69	77.5 ± 1.34
11.0	65.6 ± 4.57	71.7 ± 3.50	74.2 ± 3.75
11.5	73.8 ± 3.50	81.0 ± 4.58	80.8 ± 3.75
12.0	69.4 ± 4.96	72.1 ± 4.43	79.3 ± 2.02
12.5	71.7 ± 3.00	78.0 ± 4.64	76.7 ± 2.11
13.0	55.6 ± 3.95	60.0 ± 3.27	74.4 ± 1.76
14.0	70.4 ± 3.68	76.0 ± 6.78	75.0 ± 4.08
18.0	25.0 ± 0.00	60.0 ± 3.25	70.6 ± 3.83

Generally the normal morphology tended to decrease with an increase in age. The lowest normal morphology was 63.7 ± 6.49% on 18 h at 33 weeks of age and the highest was 87.1 ± 3.25% on 11.5 h at 45 weeks of age (Table 3.3.4.3). The same high within-treatment variation was seen in the percent normal morphology as was evident in both the concentration and motility and there was also no photoperiodic response.

Table 3.3.4.3 *Mean (±SEM) sperm morphology of male broiler breeders transferred from 8 h to various other photoperiods over time*

Final Photoperiod	Morphology		
(h)	(% normal)		
	Week 33	**Week 45**	**Week 51**
8.0	74.0 ± 1.87	64.5 ± 5.08	78.2 ± 4.22
9.0	86.7 ± 3.07	77.5 ± 4.01	67.1 ± 4.21
9.5	69.8 ± 2.79	76.4 ± 3.49	66.7 ± 5.24
10.0	81.3 ± 3.75	86.6 ± 2.83	77.3 ± 3.23
10.5	75.5 ± 2.44	74.3 ± 7.70	71.7 ± 5.85
11.0	80.6 ± 1.94	81.7 ± 2.49	71.8 ± 5.75
11.5	81.7 ± 2.20	87.1 ± 3.25	80.7 ± 2.40
12.0	75.0 ± 4.17	81.9 ± 2.34	75.1 ± 3.80
12.5	76.7 ± 2.76	66.7 ± 5.11	82.1 ± 3.01
13.0	76.8 ± 3.09	85.9 ± 1.89	70.4 ± 6.75
14.0	78.0 ± 2.55	75.6 ± 2.36	64.0 ± 4.65
18.0	63.7 ± 6.49	66.8 ± 2.99	68.0 ± 6.26

When correlations were analysed it was found that no high correlations existed between the semen characteristics, the final photoperiod and age but some interesting correlations are presented in Table 3.3.4.4. It is expected that the semen characteristics of the birds would be starting to decrease as the birds aged but the correlations indicate that the semen concentration and sperm motility increase with age while sperm morphology decreases with age. Interestingly, all of the measures of semen quality decreased with an increase in photoperiod with sperm motility being the most affected. The decrease in concentration with longer photoperiods is similar to that seen in males at female ASM (Figure 3.3.1.3).

Table 3.3.4.4 *Correlations between semen characteristics, photoperiod and age*

	Semen Concentration (# live sperm per ml semen)	Sperm morphology (% normal)	Sperm motility (% normal)
Photoperiod (h)	-0.1809	-0.1041	-0.2313
Age (weeks)	0.3370	-0.1013	0.2897

3.3.5 Sperm-egg interaction

The number of sperm trapped in the layers of the vitelline membrane of a hen's egg are likely to be representative of the number of sperm which surround the ovum at the time of fertilisation (Wishart, 1987). Furthermore, the numbers of OPL-sperm have been shown to be correlated with the proportion of uterovaginal SST containing sperm on day 14 after insemination (Brillard & Antoine, 1990) and with the number of sperm extracted from the hen's SST (Brillard, 1993). Thus the fertility of a chicken egg is a function of the number of sperm trapped, per unit area, in the OPL of the egg (Wishart, 1987).

By examining the PL of a hen's egg, the distinctive comma-shaped appearance of the fluorescing sperm nuclei could be could be clearly seen, as shown in Figure 3.3.5.1, after being stained with DAPI.
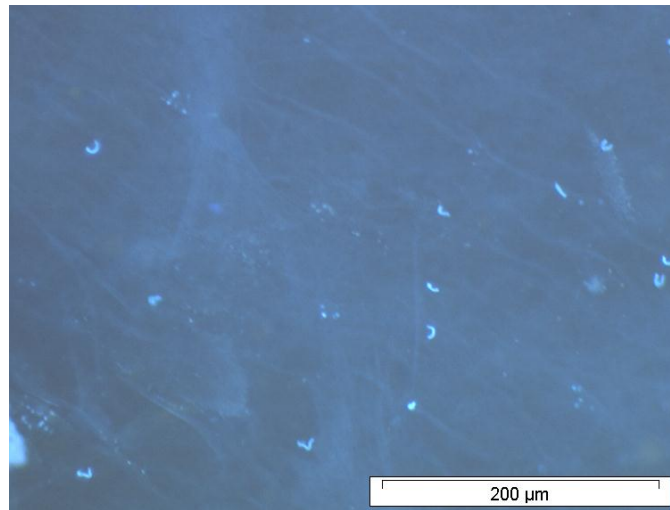
Figure 3.3.5.1 *Fluorescent sperm nuclei trapped in the perivitelline layer of a hen's egg after staining with DAPI*

The number of sperm counted per mm^2 of membrane ranged from 0 to 17 over the duration of the experiment. This result is in partial agreement to that found by Wishart (1987) where the numbers of sperm counted per mm^2 ranged from 0 to 35 depending on the number of sperm inseminated and the number of days between insemination and laying. The birds were provided with 14 h of light per day and the author did not state the age of the male birds. The difference in the numbers of sperm seen between these two trials could be ascribed to the higher concentration of semen that was inseminated into the females. In this trial a constant concentration of 30 million sperm per 50µl of diluted semen was inseminated but Wishart (1987) diluted semen containing approximately 4000 million sperm/ml 2-, 4-, 8- and 16-fold.

Wishart (1987) assumed the radius of an average egg to be 15mm resulting in the total area of the vitelline membrane to be approximately 2829mm^2. If this is the case, then the 17 sperm counted in the first fertile egg laid by one bird inseminated with 30 million sperm would result in a membrane containing approximately 48093 sperm which accounts for only 0.16% of the inseminated sperm. Wishart (1987) found that after inseminating a hen with 60 million sperm, the first egg laid contained about 0.2% of the inseminated sperm in the membrane. This figure may be dependent on the number of sperm which enter the oviduct and the capacity of the SST. After AI, less than 1% of the inseminated sperm are

selected for transport to and entry into the SST and that the sperm storage efficiency is logarithmically dependent on the number of sperm inseminated (Brillard & Bakst, 1990; Brillard, 1993). The initial quantity and quality of sperm at the time of AI directly influence the degree of selection exerted by the hen on the subsequent numbers of sperm stored in the SST (Brillard, 2003).

The trend in the numbers of OPL-sperm in the eggs laid after AI is illustrated in Figure 3.3.5.2. The numbers of OPL-sperm decrease as the length of time between insemination and oviposition is increased (Figure 3.3.5.2 a). The minimum number of sperm required for an egg to have a high probability of being fertile, based on the numbers of OPL-sperm as described by Brillard and Antoine (1990) (0.43 sperm/mm^2) and Wishart (1997) (>3 sperm/mm^2) are shown in Figure 3.3.5.2 a. The number of sperm in consecutive eggs from a typical hen decreased logarithmically in response to the number of days following insemination (Figure 3.3.5.2 b). This result is in agreement with that found by Wishart (1987). Depending on the age of the males in this trial, it was found that by either day 8 or 13 no sperm was present in the OPL of the eggs. This finding is in agreement with that of Gumułka and Kapkowska (2005) who stated that the effective duration of fertility (the number of days after insemination during which a hen lays fertile eggs until the first infertile egg) was 13.8 ± 0.22 days and Brillard and Antoine (1990) who found that numbers of sperm decreased gradually until day 13 after insemination.
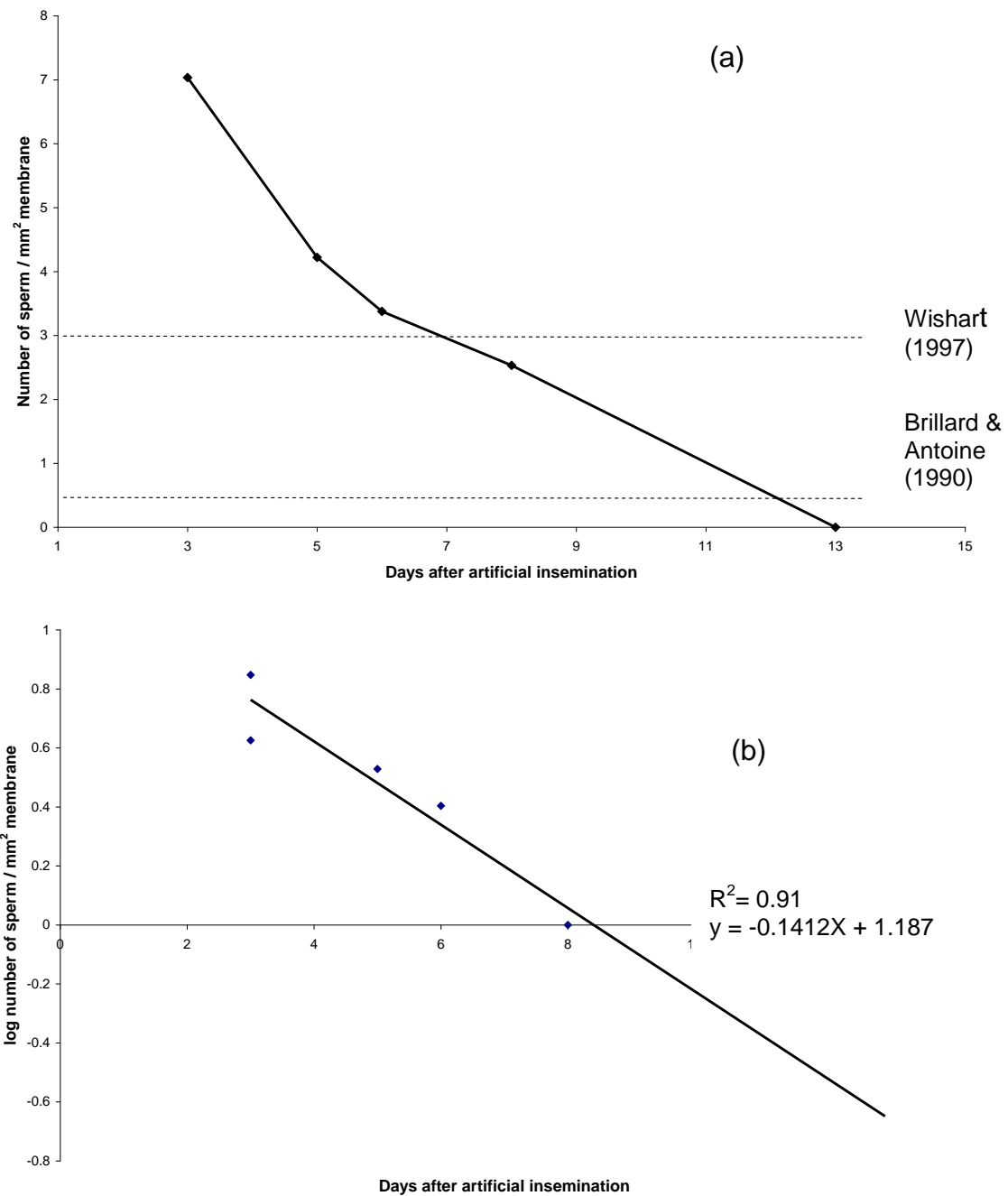
Figure 3.3.5.2 *The decline in the number of sperm (and the minimum levels for fertility) (a) and log number of sperm (b) trapped in one mm^2 of the outer perivitelline membrane of eggs laid by a typical female (at 40 weeks) as the number of days after artificial insemination increase*

When comparing the results obtained by calculating the fertility status of eggs based on the number of sperm required to be trapped in the vitelline layer as stated by Wishart (1997) and Brillard and Antoine (1990), it was calculated that at 40 weeks of age (across all days post AI), 19.08% of the eggs evaluated were fertile based on Wishart (1997) and 74.80% based on Brillard and Antoine (1990). At 45 weeks of age (across all days post AI) these numbers decreased to 4.08% and 63.52% respectively and by 51 weeks of age (across all days post AI), 1.81% and 52.89% respectively. Thus, there was a large discrepancy in the percentage of fertile eggs based on the two methods of calculation.

The analysis of the data using the calculation in Wishart (1997) showed that the age of the male, the number of days that had elapsed between AI and oviposition and the final photoperiod all had a significant effect on the fertility of the eggs produced. There were no significant interaction effects. It was found that at an age of 45 and 51 weeks, sperm numbers in the OPVL were significantly lower ($p<0.01$) than at 40 weeks. As the number of days post AI increased, the fertility of the egg decreased and there was a significant difference ($p<0.01$) between day 4 and days 6, 8 and 13 post AI. The only photoperiods which had a significant effect when compared with 8 h were 12 h ($p<0.05$) and 13 h ($p< 0.05$). The probability of obtaining a fertile egg under different photoperiods and different days post AI are illustrated in Figure 3.3.5.3, but more detailed probabilities are given in Appendix 9. As can be seen in Figure 3.3.5.3 a, the highest probability of an egg being fertile is 4 days post AI when the males have been exposed to 8 h of light. This probability decreases with an increase in both photoperiod and days post AI. As the male ages, the probability of an egg being fertile also decreases from approximately 80% to under 15% at 45 and 51 weeks of age (Appendix 9). Across all age groups, there is a low probability of an egg being fertile.

Figure 3.3.5.3 *The probability of an egg being fertile on different photoperiods and days post insemination at 40 weeks (a), 45 weeks (b) and 51 weeks (c) calculated using >3 sperm/mm² as the minimum number of sperm required to be trapped in the vitelline layer for an egg to fertile as described by Wishart (1997)*

When the number of OPL-sperm, described by Brillard and Antoine (1990), was used to calculate the fertility status of a hens egg, it was found that the results followed the same trends as those found when using the calculation from Wishart (1997) but the differences were more distinct. Age of the male, the number of days post AI and the photoperiod all had significant effects on the fertility of the egg and there were no significant interaction effects. However, unlike the results found when using the calculation from Wishart (1997), the fertility of the egg was not significantly different when the males were 40 and 45 weeks of age but the fertility of the egg was significantly less than at 40 weeks when the males reached 51 weeks of age ($p<0.01$). Day 4 and 6 after AI did not differ in their effects on the fertility of the egg but the probability of a fertile egg was significantly lower days 8 ($p<0.01$) and 13 ($p<0.01$) post AI. The photoperiods which differed significantly from, and were lower than, 8 h were 9, 10 and >12 h. This would suggest that there is no photoperiodic response except a general decline with longer photoperiods. These results are illustrated in Figure 3.3.5.4 and more detailed probabilities can be found in Appendix 9. At 40 weeks, the trend seen for the probability of an egg being fertile was higher than at 45 or 50 weeks and the photoperiod had a negative effect on the probability in that the longer the photoperiod, the lower the probability of an egg being fertile. This photoperiodic trend was similar at 45 weeks of age but at 51 weeks the trend was inversed and the longer photoperiods tended to have a higher probability of an egg being fertile, particularly as the number of days after insemination increased. This was an unexpected result and cannot be explained. An interesting trend is seen as the male birds age in that the probability of a fertile egg drops drastically from 40 to 45 and 51 weeks with an increase in the days post AI. At 40 weeks the probability of a fertile egg does not fall below 55% regardless of photoperiod or days post AI. This is possibly due to the physiological changes in and causing a decreased capacity of the SST as hens age (Pierson *et al.*, 1988).

The probability of an egg being fertile is strongly dependant on the number of OPL-sperm. This has been demonstrated by the probabilities calculated using the different numbers of Wishart (1997) and Brillard and Antoine (1990). When only half a sperm is required to be trapped per mm^2 of membrane (Brillard & Antoine,1990) there is a higher probability of an egg being fertile than when > 3 sperm need to be trapped per mm^2 of membrane (Wishart, 1997). This comparison questions the actual number of OPL-sperm that are required to be

trapped in the OPL for an egg to have a high probability of being fertile. It is not surprising that using the calculation from Wishart (1997) in which a higher number of OPL-sperm are required, that the probability of an egg being fertile declines. The probability of an egg being fertile is higher using the calculation from Brillard & Antoine, (1990) where 0.43 sperm/mm^2 equates to a fertile egg allows a greater proportion of fertile eggs than the calculation from Wishart (1987). When predicting the probability of an egg being fertile, it may be better to use a higher number of OPL-sperm as the eggs calculated to have a high probability would be more likely to be correct rather than overestimating the probabilities when a low number of OPL-sperm is used. However, the trends in fertility with days post AI and photoperiod are more obvious using the method of Brillard & Antoine (1990). When the calculated probabilities were compared to a primary breeders hatchability curve (Cobb, 2005), it was found that the probabilities calculated using the method of Brillard & Antoine (1990) were similar to those of the hatchability curve whereas the fertility probabilities for the Wishart (1997) calculation were drastically lower. Thus the method of Brillard & Antoine (1990) may be a better indicator of hatchability than the Wishart (1997) method.

To date there is no information available on the effect of the photoperiod provided to the male on the number of OPL-sperm trapped in a hen's egg and this trial appears to be the first in this field. Studies evaluating the effect of broiler breeder age on the fertility of eggs are more focused on the effect of the age of the female as the age related decrease in fertility is more pronounced in the female when compared with the male due to the efficiency of the SST decreasing (Bramwell *et al.*, 1996; Gumułka & Kapkowska, 2005). Bramwell *et al.* (1996) reported a significant increase in sperm penetration of the PL and percentage fertile eggs in both young and old females inseminated with semen from old verses young males. All birds were on a 17 h photoperiod and this is therefore contradictory to the results from this experiment, where on a long photoperiod, older males showed a decrease in the number of OPL-sperm.

At 51 weeks of age, the numbers of OPL-sperm could not be accurately predicted by the area of the comb, the sperm motility or morphology or the number of rounded or sickle primary wing feathers, as there were no significant effects of any of these measures on OPL-sperm.
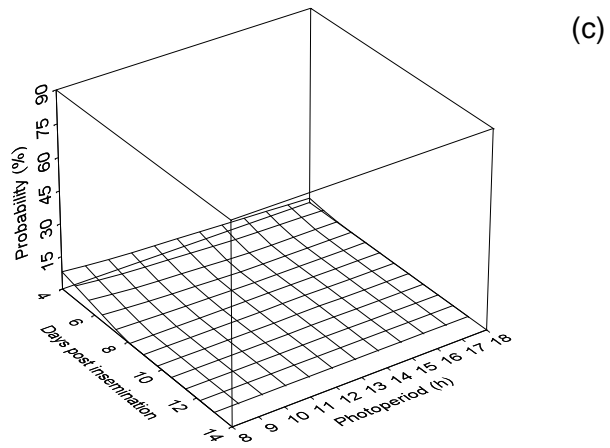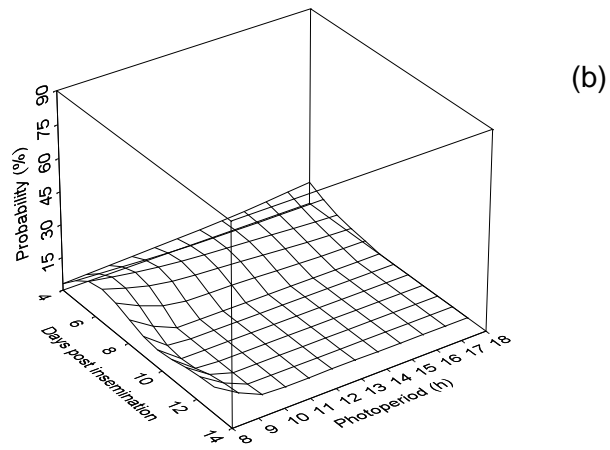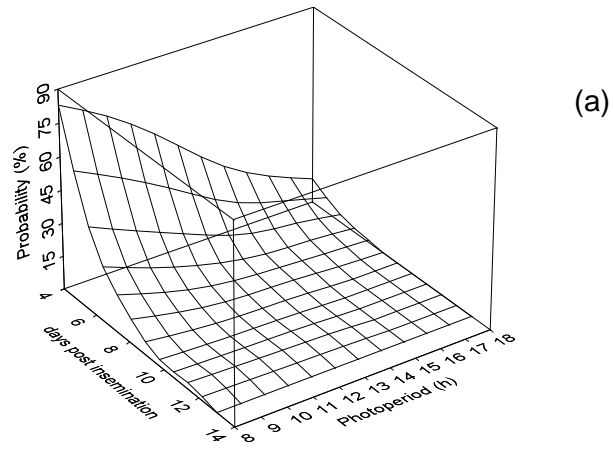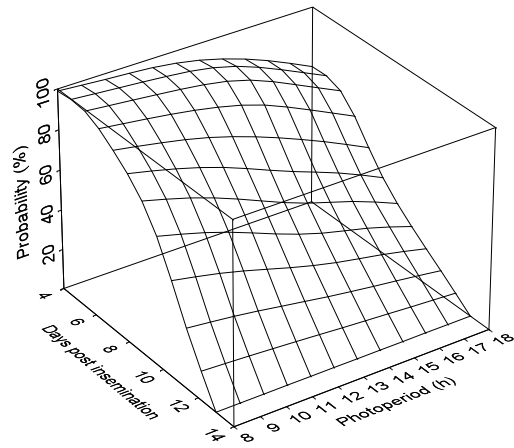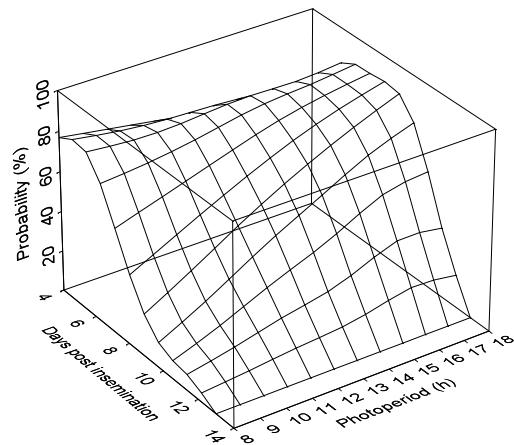
Figure 3.3.5.4 *The probability of an egg being fertile on different photoperiods and days post insemination at 40 weeks (a), 45 weeks (b) and 51 weeks (c) calculated using 0.43 sperm/mm^2 as the minimum number of sperm required to be trapped in the vitelline layer for an egg to fertile as described by method described by Brillard and Antoine (1990)*
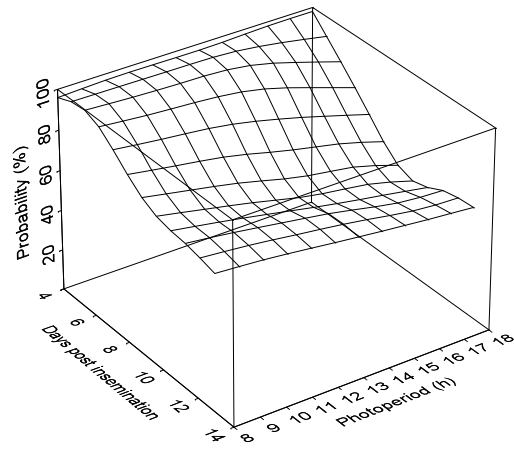
3.4 CONCLUSIONS

Male broiler breeders did not respond to photostimulation in the same manner as the females did. The ASM of the males was not affected by the final photoperiod which suggests that the males could be photostimulated at an earlier age in order to try and elicit a photosexual response. At the time of female sexual maturity, sperm motility and morphology, testes weights and comb area of the male were not significantly affected by the final photoperiod. However, both testosterone and semen concentration were significantly affected by photostimulation in that testosterone was higher on longer photoperiods and semen concentration was higher on shorter photoperiods.

The testosterone concentration did not affect the ASM of the male broiler breeders but this may due to testosterone concentration only increasing one to four weeks after an increase in LH. An increase in LH would probably have been seen around 20 weeks of age and thus testosterone would have only increased after approximately 22 weeks of age which it did. Testosterone concentration significantly affected the area of the comb of the male but a plateau was reached after which comb area was no longer affected. Plasma testosterone levels were found to be positively correlated with age and with sperm motility at 30 weeks of age.

At 51 weeks of age, the final photoperiod did significantly affect the average testes weights where males on photoperiods >13 h had lower testes weights than males transferred to shorter photoperiods. Some males had undergone testes regression and were not producing semen. It appears that some males may undergo adult photorefractoriness, signalled by testes regression, yet two males which had regressed testes were still producing semen, so it may have been too early to determine. The comb area of the birds was positively correlated with age and was affected to an extent by the final photoperiod but was not correlated with semen characteristics. An interesting finding was the regression of the comb in some male birds. It was found that sickled feathers were moulted and replaced by rounded feathers but the photoperiod did not affect the numbers of feathers. Semen characteristics (concentration, motility and morphology) were not significantly affected by the final photoperiod.

The probability of an egg being fertile was affected by the age of the male, the number of days lapsed between AI and oviposition, and the final photoperiod that the male was maintained on. However the probability was also affected by the method used to determine the fertility of an egg from the number of OPL-sperm. The method from Brillard & Antoine (1990), which required less OPL-sperm to be fertile, resulted in higher probabilities of fertility, regardless of treatment.

The critical and saturation daylengths in female broiler breeders have been defined by Lewis *et al.* (2008) but information regarding these two daylengths in males has not been reported. From the findings in this trial, it appears that when males are reared on 8 h they do not have a critical daylength as these birds still respond irrespective of a lack of photostimulation. The saturation daylength seems to be roughly similar to that of the females (around 13 h) based on the evidence provided by the regression of the testes and a lower semen concentration on photoperiods >13 h.

In conclusion, the males did not respond to photostimulation to the same degree as the females, which appears to be a common trend of male animals. A large amount of variation was found in the response of ASM to photoperiod, which could be not be ascribed to variation in body weight and was therefore assumed to be of genetic origin, and could be due to the lack of selection of males for reproductive traits. The use of standard semen analysis methods becomes questionable as a reliable indicator of male fertilising ability but rather more reliable and objective tests (such as sperm-egg interactions, computer assisted sperm analysis) should be used to assess the fertility of male birds. Secondary sexual characteristics may indicate the testosterone levels in the bird but did not indicate the fertility of the male in this experiment.

CHAPTER 4

General Discussion

The fertility of broiler breeder flocks is generally accepted to be low. However, the problem faced by the poultry industry is that the majority of experiments are concerned with the egg laying abilities of the female and egg fertility which also measures a female contribution. Considering that the phenotype of the bird is an expression of the effects of the genotype and environment, it seems logical that if the genotype cannot be changed without compromising the required broiler traits, then the environment could be altered to maximise fertility. Thus, the aim of this trial was to determine the effects of different degrees of photostimulation on the ASM and subsequent fertility of male broiler breeders in the hopes of increasing the flock fertility through management of the male.

Male ASM was not influenced by the final photoperiod applied in this trial. This suggests that males can be photostimulated at an earlier age, before their female counterparts, in order to bring about sexual maturity earlier. The fact that the males matured without photostimulation suggests that 8 h is sufficient for males to mature. However, common practice is to rear the sexes on the same lighting schedule. If this is the case, given that females achieve a maximum advance in AFE by the transfer of 8 to 13 h of light and that photoperiods longer than 13 h of light produce no further advance (Lewis *et al*., 2008), it becomes apparent that providing broiler breeder flocks with photoperiods longer than 13 h would be unnecessary and may even be detrimental to fertility as male semen concentration would be low at the time females start egg production. Lewis *et al.* (2008) found that transferring females from 8 to 11 h at 20 weeks of age resulted in the first significant increase in LH and statistically earlier ASM. Based on this information, it becomes evident that if male and female broiler breeders are to be reared under the same photoschedule, on an 8 h photoperiod with an increase at 20 weeks, this increase should be between 11 and 13 h as this would result in some advance in female AFE while at the same time increasing male semen and testosterone concentrations to coincide with the females becoming sexually mature. Thus, the recommended, and currently used, lighting programmes for broiler breeders are correct concerning the increase from 8 h to 11-12 h at

20 weeks but the use of longer photoperiods such as a 16 h photoperiod may lead to a decreased male fertility in the flock earlier in the laying period and need to be revised.

When considering photostimulating males at an age earlier than 20 weeks, the effects of juvenile photorefractoriness need to be considered. Unpublished data, in which broiler breeder pullets were grown to reach 2.1 kg at 20 weeks and transferred from 8 to 16 h of light at different ages (10 to 25 weeks), suggests that in a control-fed flock, juvenile photorefractoriness is not completely dissipated until around 18 weeks of age (Lewis & Morris, 2006). Further unpublished data suggested that feed control to maintain body weights may alter the rate at which juvenile photorefractoriness is dissipated in broiler breeder pullets, in that pullets, grown to 2.1 kg at 20 weeks, did not fully respond to photostimulation until about 18 weeks but when the feed control was relaxed, and the birds reached 2.1 kg in 15 weeks, these birds responded to photostimulation from 14 weeks (Lewis & Morris, 2006). Tyler and Gous (2008) found that all male broiler breeders reared on 8 h should respond to photostimulation at 14 weeks. Considering that the males in this trial did not respond to photostimulation at 20 weeks and that some males produced semen by 21.2 weeks and that spermatogenesis takes 15 days, it was assumed that the males had begun to mature before photostimulation. This result, combined with the unpublished data, suggests that males may dissipate juvenile photorefractoriness before females. This would be expected as males generally mature earlier than females to ensure that when the first female in the flock reaches sexual maturity, the male is already mature and able to fertilise the female (Raitt & Ohmart, 1966; Rosa & Bryant, 2003). A practical implication for males maturing on a rearing photoperiod of 8 h would be the possibility of placing day old male chicks into the female house when the females are approximately 4 weeks. The males would mature on 8 h and then respond to the photostimulation (provided that the <13 h is used) at 16 weeks resulting in better semen quality. This would also help save on male feed costs.

Current practice in the Midlands area of KwaZulu-Natal is to rear male and female broiler breeder chicks in the same house under the same lighting conditions and to separate the sexes half way through the rearing period in order to provide the females with more space. The males are often placed in an open sided house which, depending on the season, provides the male with a greater light intensity and daylength (T. Nixon, 2008, Pers.

Comm., Meadow Feeds, P.O. Box 426, Willowton, Pietermaritzburg, 3200). The farmers following this practice have claimed that it has produced good results and the fertility of the birds has increased. In essence, providing the birds with this type of lighting programme is the same as photostimulating the birds at a younger age. However, the increase in fertility that results may be linked to the "male effect" where females are stimulated to reach sexual maturity after exposure to males close to the expected ASM. Post-weaning anoestrus in pigs may be overcome by exposure of the sow to the boar (Pearce & Pearce, 1992) and the "ram effect" is a technique available to manipulate reproduction in females during the anoestrous period (Rosa & Bryant, 2002).

Testosterone levels in this trial had no significant effect on the ASM of the males. A better measure of ASM is LH. However, in this trial, the measurement of LH, although attempted, was not successful. Testosterone did have an effect on the area of the comb. Thus males with larger combs had a higher concentration of testosterone but only up to a certain point. It may be assumed that comb area may then be a good indicator of testosterone levels in a male. Considering that testes sizes and weights were not examined during the trial except upon slaughter, it is not possible to say if testosterone may indicate the size of the testes or furthermore if comb area may indicate testes size or weight. Unexpectedly, considering that comb area is known to be a testosterone-mediated trait and that sperm maturation is also testosterone mediated, no relationship existed between semen characteristics and comb area. This may be in agreement with a number of authors (Burrows & Titus, 1939; Pizzari *et al*., 2004; and Bilcik *et al*., 2005) but disagrees with McGary *et al*., (2002). Based on their findings, Pizzari *et al*. (2004) proposed that male fertilising efficiency consists of two independent events, one being gonadal investment which can be predicted by comb size and the second being determined by sperm quality. These results agree with this statement, in that sperm quality can not be assessed by the area of the comb, but it may be assumed that testes weights can be predicted by the area of the comb (Tyler and Gous, 2006), although this has only been determined in young birds.

At 51 weeks of age, the final photoperiod did have a significant effect on the average testes weights of the males where males on longer photoperiods (> 13 h) had lower testes weights than males on shorter photoperiods (≤ 13 h). It is well known that adult

photorefractoriness is induced by prolonged exposure to long photoperiods and that photorefractoriness is characterised by the regression of the gonads (Dawson, 2001; Dawson *et al.*, 2001). Based on these results, the evidence of testis regression upon slaughter and the literature, it would seem appropriate to assume that the birds on the longer photoperiods were becoming photorefractory. Considering that some males had undergone testes regression but were still producing semen while others had stopped producing semen suggests that some males may have been relatively photorefractory while others showed absolute photorefractoriness, and the remaining birds were still photosensitive. There is no literature available to support or reject this hypothesis. In view of the fact that male sheep have the ability to decrease testes weight and sperm production without the cessation of spermatogenesis and sexual activity (Rosa & Bryant, 2003), it would be interesting to ascertain whether some male broiler breeders have the same ability. It may also have been too early towards the end of the breeding season to observe the effect of photoperiod on the decline of fertility as commercial broiler breeder parent stock are kept until 60 weeks.

Semen characteristics after 33 weeks of age were not affected by the degree of photostimulation applied. The ability of these assessments to determine fertility or fertilising potential is still controversial and this controversy initiated the development of tests which evaluate the actual fertilising ability of an individual's semen. By assessing the number of OPL-sperm trapped in a hen's egg, the probability of the egg being fertile was calculated. It was found that the age of the male, the number of days between insemination and the laying of the egg and photoperiod all had a significant effect on the probability of an egg being fertile. Depending on the number of sperm tapped per mm^2 of membrane that is used as a baseline to distinguish between fertile or unfertile, the probability of a fertile egg changes. It is questionable as to what this baseline number should be as if it is too low, the probability may be overestimated but if the number is too high the probability may be underestimated. To date there is no information available on the effect of the photoperiod provided to the male on the number of OPL-sperm trapped in a hen's egg and this trial appears to be the first in this field.

A large amount of variation existed in the response of individual males to photoperiod after stimulation. It was concluded that this variation was probably of a genetic origin due to all

males on a specific treatment being exposed to the same environmental conditions. The only explanation for genetic variation, in a line so uniform for production traits, is that of the lack of selection pressure in male broiler breeders for sexual traits. Females are selected for egg laying and hatchability (which would also require male fertility) however male selection is biased towards broiler characteristics (J. McAdam, 2008, Pers. Comm., Breeding Programme Director, Aviagen Ltd, UK), such as growth and carcass yield, which are negatively correlated with fertility (Sexton, 1983; Fontana *et al.*, 1990; Aviagen, 2001; Ciacciariello & Gous, 2005; Cobb, 2005; Lewis, 2006).

The findings of this study indicate that more research is required in the area of male fertility and the effects of lighting thereon. It has been shown that males respond differently to light when compared with females and thus consideration needs to be given to this point when lighting programmes are designed.

4.1 Conclusions

The main findings of this trial were that male broiler breeders respond to light in a different manner when compared with females and that individual males vary greatly in their sexual responses and characteristics due to a lack of selection for sexual traits. Despite the information that this trial has contributed to the knowledge regarding the effects of different degrees of photostimulation on male broiler breeders, there are still areas which need further research.

It would be beneficial to the industry to examine the effects of photostimulating male broiler breeders at a younger age. Information would need to be gained regarding the age at which juvenile photorefractoriness is dissipated and what degree of photostimulation is needed to dissipate photorefractoriness in the shortest period of time. Reducing the ASM in males may be beneficial as it would allow more time for the male to mature and for semen characteristics to improve before the female matures and needs to be fertilised. This may help increase the number of chicks hatched if the flock fertility is improved early in the laying period.

In order to determine the exact ASM in males, LH assays should be performed. Generally the age at first semen production is given as the ASM, however, males may be capable of producing semen but do not respond favourably to manual semen collection techniques.

The use of secondary sexual characteristics as indicators of male fertility is still a contentious topic and requires further research.

Considering that adult photorefractoriness terminates the production of sperm and eggs, it would be valuable for researchers to determine the nature, degree and causes of adult photorefractoriness in male broiler breeders.

The critical and saturation daylengths for females have been evaluated but for males this requires furthur research. It has been suggested here that males reared on an 8 h photoperiod do not have a critical daylength and that the saturation daylength is similar to that of females which is around 13 h. This research could be furthered to accurately determine the critical and saturation daylengths for male broiler breeders.

Due to the negative genetic correlation between growth and fertility, it would be beneficial to investigate the possibility of selecting males for certain fertility traits in order to improve flock fertility. By identifying the males which are higher producers and have more sperm trapped in the perivitelline layer of an egg and selecting these birds, it may be possible to reduce the genetic variation and improve male fertility.

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APPENDICES

Appendix 1

Formula used to calculate the predicted testis weight from comb area

Predicted testis weight= -3.54 + 25.16 / (1 + exp (-0.0877 * (A – 25.76)))

Where:

 A= Comb area   (e.g. comb area of 16.5 cm^2)

Example

-3.54 + 25.16 / (1 + exp (-0.0877 * (16.5 – 25.76))) = 4.19g

The predicted weight of 1 testis is 4.19g when the comb area is 16.5 cm^2

Appendix 2

Schedule for semen analysis related to the age at first egg of the females

Final photoperiod	Female mean age at first egg	Male semen analysis
(hours)	(days)	(days)
8.0	200	198
9.0	198	196
9.5	195	193
10.0	198	196
10.5	192	190
11.0	185	183
11.5	186	184
12.0	180	178
12.5	180	178
13.0	183	181
14.0	182	180
18.0	177	175

Appendix 3

Composition of Eosin and Nigrosin solution.

- 8ml distilled water
- 3 drops Eosin*
- 10 drops Nigrosin**

*Eosin- 5g/100ml distilled water

** Nigrosin- 10g/100ml distilled water

Appendix 4

Composition of Tyrodes solution

Component	Quantity
	(g)
CaCl2	0.2*
MgCl26H2O	0.1
KCl	0.2
NaHCO3	1.0
NaCl	8.0
NaH2PO4	0.04 anhydrous/ 0.052 hydrous
glucose	1.0
Distilled water	up to 1000ml

* add last while stirring to prevent precipitation

The solution should be filtered into a sterile container and stored in the refrigerator.

Appendix 5

Formula used to calculate the concentration of live sperm per ml of semen

Concentration= ((A / B) / C) x 1000* x D x E

Where:

- A- Total number of sperm counted   (e.g. 106 sperm in 20 blocks)
- B- Total number of blocks counted   (e.g. 20 blocks)
- C- Volume   (e.g. $0.00025mm^3$)
- D- Dilution factor   (e.g. 50µl sperm in 8.0ml Eosin and

               Nigrosin solution gives a factor of 160)
- E- % live sperm   (e.g. 90%)

*Conversion factor to convert the volume from mm^3 to ml

<u>Example</u>

(106 / 20) / 0.00025) x 1000 x 160 x 0.9 = 3053 million live sperm per ml of semen

Appendix 6

Formula used to calculate the amounts of semen and Tyrodes Solution needed for artificial insemination

Tyrodes= ((A / (B / C x 0.001*)) x ((D – (B / C x 0.001))

Where:

A- Microliteres of semen collected   (e.g. 100µl of semen)

B- Required concentration of sperm for A.I.   (e.g. 30 million sperm)

C- Concentration   (e.g. 3053 million live sperm per

                                    ml of semen)

D- Required volume for A.I.   (e.g. 50µl)

*Conversion factor to convert the volume form ml to µl

<u>Example</u>

((100 / (30×10^6 / 3053×10^6 x 0.001)) x ((50 - (30×10^6 / 3053×10^6 x 0.001)) = 408.8µl of Tyrodes solution to be mixed with the 100µl of semen in order to A.I. 50µl which contains exactly 30 million sperm

Appendix 7

Composition of the fluorescent stain 4,6-diamidino-2-phenylindole in PBS (DAPI)

Component	Quantity (g)
NaCl	8.0
KCl	0.2
Anhydrous Na_2HPO_4	1.2
Anhydrous KH_2PO_4	0.2
Distilled water up to 1000ml	

The solution should be filtered into a sterile container and stored in the freezer under aluminium foil to avoid exposure to light.

Appendix 8

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. 26 sperm)

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

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

<u>Example</u>

26 / 3553743.80 x 1 000 000 = 7 sperm per mm^2

Appendix 9

Tables showing the probability of a fertile egg being laid as effected by the age of the bird, the number of days post artificial insemination, the photoperiod and the method used to determine fertility

Age (weeks)	Days post A.I. (days)	Photoperiod (h)	Probability		Age (weeks)	Days post A.I. (days)	Photoperiod (h)	Probability
			Wishart (1997)					Brillard and Antoine (1990)
	4	8.0	0.83			4	8.0	0.96
		9.0	0.77				9.0	0.96
		9.5	0.74				9.5	0.97
		10.0	0.70				10.0	0.97
		10.5	0.66				10.5	0.97
		11.0	0.62				11.0	0.97
		11.5	0.57				11.5	0.97
		12.0	0.53				12.0	0.97
		12.5	0.48				12.5	0.97
		13.0	0.43				13.0	0.97
		14.0	0.34				14.0	0.97
		18.0	0.11				18.0	0.98
	6	8.0	0.31			6	8.0	0.99
		9.0	0.25				9.0	0.99
		9.5	0.22				9.5	0.99
		10.0	0.20				10.0	0.98
		10.5	0.18				10.5	0.98
		11.0	0.16				11.0	0.98
		11.5	0.14				11.5	0.97
		12.0	0.12				12.0	0.97
		12.5	0.10				12.5	0.96
		13.0	0.09				13.0	0.95
		14.0	0.07				14.0	0.94
40		18.0	0.02		40		18.0	0.79
	8	8.0	0.11			8	8.0	0.85
		9.0	0.08				9.0	0.83
		9.5	0.07				9.5	0.82
		10.0	0.06				10.0	0.81
		10.5	0.05				10.5	0.80
		11.0	0.05				11.0	0.79
		11.5	0.04				11.5	0.78
		12.0	0.03				12.0	0.77
		12.5	0.03				12.5	0.75
		13.0	0.02				13.0	0.74
		14.0	0.02				14.0	0.71
		18.0	0.00				18.0	0.58
	13	8.0	0.00			13	8.0	0.68
		9.0	0.00				9.0	0.67
		9.5	0.00				9.5	0.66
		10.0	0.00				10.0	0.66
		10.5	0.00				10.5	0.65
		11.0	0.00				11.0	0.64
		11.5	0.00				11.5	0.64
		12.0	0.00				12.0	0.63
		12.5	0.00				12.5	0.62
		13.0	0.00				13.0	0.62
		14.0	0.00				14.0	0.60
		18.0	0.00				18.0	0.55

Wishart (1997)					Brillard and Antoine (1990)			
Age (weeks)	Days post A.I. (days)	Photoperiod (h)	Probability		Age (weeks)	Days post A.I. (days)	Photoperiod (h)	Probability
45	4	8.0	0.03		45	4	8.0	0.96
		9.0	0.04				9.0	0.96
		9.5	0.04				9.5	0.97
		10.0	0.04				10.0	0.97
		10.5	0.04				10.5	0.97
		11.0	0.05				11.0	0.97
		11.5	0.05				11.5	0.97
		12.0	0.05				12.0	0.97
		12.5	0.05				12.5	0.97
		13.0	0.06				13.0	0.97
		14.0	0.06				14.0	0.97
		18.0	0.10				18.0	0.98
	6	8.0	0.15			6	8.0	0.99
		9.0	0.13				9.0	0.99
		9.5	0.12				9.5	0.99
		10.0	0.11				10.0	0.98
		10.5	0.10				10.5	0.98
		11.0	0.09				11.0	0.98
		11.5	0.09				11.5	0.97
		12.0	0.08				12.0	0.97
		12.5	0.07				12.5	0.96
		13.0	0.07				13.0	0.95
		14.0	0.06				14.0	0.94
		18.0	0.03				18.0	0.79
	8	8.0	0.12			8	8.0	0.85
		9.0	0.00				9.0	0.83
		9.5	0.00				9.5	0.82
		10.0	0.00				10.0	0.81
		10.5	0.00				10.5	0.80
		11.0	0.00				11.0	0.79
		11.5	0.00				11.5	0.78
		12.0	0.00				12.0	0.77
		12.5	0.00				12.5	0.75
		13.0	0.00				13.0	0.74
		14.0	0.00				14.0	0.71
		18.0	0.00				18.0	0.58
	13	8.0	0.12			13	8.0	0.68
		9.0	0.00				9.0	0.67
		9.5	0.00				9.5	0.66
		10.0	0.00				10.0	0.66
		10.5	0.00				10.5	0.65
		11.0	0.00				11.0	0.64
		11.5	0.00				11.5	0.64
		12.0	0.00				12.0	0.63
		12.5	0.00				12.5	0.62
		13.0	0.00				13.0	0.62
		14.0	0.00				14.0	0.60
		18.0	0.00				18.0	0.55

Wishart (1997)				Brillard and Antoine (1990)			
Age (weeks)	Days post A.I. (days)	Photoperiod (h)	Probability	Age (weeks)	Days post A.I. (days)	Photoperiod (h)	Probability
51	4	8.0	0.08	51	4	8.0	0.77
		9.0	0.07			9.0	0.75
		9.5	0.07			9.5	0.74
		10.0	0.06			10.0	0.73
		10.5	0.06			10.5	0.72
		11.0	0.06			11.0	0.71
		11.5	0.05			11.5	0.70
		12.0	0.05			12.0	0.69
		12.5	0.05			12.5	0.68
		13.0	0.04			13.0	0.67
		14.0	0.04			14.0	0.65
		18.0	0.02			18.0	0.57
	6	8.0	0.04		6	8.0	0.77
		9.0	0.02			9.0	0.78
		9.5	0.02			9.5	0.78
		10.0	0.02			10.0	0.78
		10.5	0.01			10.5	0.79
		11.0	0.01			11.0	0.79
		11.5	0.01			11.5	0.80
		12.0	0.01			12.0	0.80
		12.5	0.01			12.5	0.81
		13.0	0.00			13.0	0.81
		14.0	0.00			14.0	0.82
		18.0	0.00			18.0	0.85
	8	8.0	0.00		8	8.0	0.38
		9.0	0.00			9.0	0.44
		9.5	0.00			9.5	0.47
		10.0	0.00			10.0	0.50
		10.5	0.00			10.5	0.53
		11.0	0.00			11.0	0.56
		11.5	0.00			11.5	0.60
		12.0	0.00			12.0	0.62
		12.5	0.00			12.5	0.65
		13.0	0.00			13.0	0.68
		14.0	0.00			14.0	0.73
		18.0	0.00			18.0	0.88
	13	8.0	0.00		13	8.0	0.00
		9.0	0.00			9.0	0.00
		9.5	0.00			9.5	0.00
		10.0	0.00			10.0	0.00
		10.5	0.00			10.5	0.00
		11.0	0.00			11.0	0.00
		11.5	0.00			11.5	0.00
		12.0	0.00			12.0	0.00
		12.5	0.00			12.5	0.00
		13.0	0.00			13.0	0.00
		14.0	0.00			14.0	0.00
		18.0	0.00			18.0	0.00