CHARACTERIZATION AND CRYOPRESERVATION OF SEMEN OF FOUR SOUTH AFRICAN CHICKEN BREEDS

by

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DECLARATION

I hereby declare that this dissertation submitted by me to the University of the Free State for the degree, **Magister Scientiae Agriculturae,** is my own independent work and has not previously been submitted for a degree to any other university. I furthermore cede copyright of this thesis in favour of the University of the Free State.

Thatohatsi Madaniel Bernice Mosenene

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List of Abbreviations

AI	Artificial Insemination
ARC	Agricultural Research Council
BPSE	Beltsville Poultry Semen Extender
Ca	Calcium
CO_2	Carbon dioxide
CASA	Computer assisted sperm analyzer
DMF	Dimethylformamide
DMA	Dimethylacetamide
DMSO	Dimethylsulfoxide
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin releasing hormone
ICSH	Interstitial cell stimulating hormone
K^+	Potassium
LH	Luteinizing hormone
mBPSE	modified Beltsville Poultry Semen Extender
Na^{++}	Sodium

NaCl	Sodium Chloride
O_2	Oxygen
SST	Sperm storage tubules
SQI	Sperm quality index

Chapter 1

General Introduction

South Africa‘s broiler industry currently produces on average 13.8 million broilers per week, while the domestic demand is growing at approximately 7% per annum. The South Africa import of poultry meat is currently approximately 10% to 20% of the consumption which demonstrates a substantial under-supply (USDA, 2007). Poultry production also constitutes an important component of the agricultural economy in the developing countries, and the industry has exploded, much greater when compared to the ruminant and pig industry. Commercial and small scale broiler production units also contribute in supplying local people with additional income and a supply of high quality protein for the household. Household poultry production is also valued in the religious and the socio-cultural lives of different cultures. However, there are certain constraints regarding the development of aspects such as disease, breeding, nutrition and marketing (Branckaert & Queye, 1995).

Poultry farming has become especially popular, due to the quick cash return, low capital imputs and subsequent poverty alleviation and income generation in the rural poor communities. Profitable poultry farming depends on quality chicks, feeds and good management. To produce enough chickens for the increasing demand, healthy broiler chicks and layers must be produced. Many commercial poultry farms rear their own parent stock and the use of artificial insemination (AI) could reduce management costs. This technique of AI has the advantage that one cockerel can be used to inseminate 20 to 30 hens, while in natural mating one cockerel only services 8 to 10 hens per day. The focus of the commercial poultry industry is thus the production of meat and eggs under intensive husbandry practices. Turkeys are however generally kept separately from males and reproduction is performed by AI, unlike in chickens where reproduction is by natural mating. This has stimulated a substantial

scientific research effort in the chicken industry (Islam *et al.,* 2002). Hens can lay a series of fertilized eggs over a period of 3 or more weeks following a single insemination (Froman & Feltmann, 2005).

White meat is currently the most preferred source of animal protein around the world (for health reasons) and it has the advantage of being accepted by most religions. This is due in part to the fact that red meat is too expensive and has certain negative health connotations. So for example saturated animal fat in red meat is said to contribute to heart attacks and arthrosclerosis. Recent research has also shown that red meat consumers face twice the risk of colon cancer. Red meat is also thought to increase the risks of rheumatoid arthritis (Yang *et al*., 2002).

Chicken producers over the years have used genetic selection and improved nutritional management practices and there has been a steady and rapid increase of the growth rate in chicken production. This has resulted in an extremely rapid growth of chicken production, which has certain detrimental effects on reproduction (Bramwell, 2002). Due to the sharp increase in chicken meat consumption it has also become important to increase the production of layers to meet the demand. Assisted reproduction technologies (ART's), such as AI which encompasses the AI deposition of semen in the hen's reproductive tract may contribute to increase poultry production, as it allows a wider use of genetically superior cockerels with a high productive performance. On the other hand ART's have the potential benefit of allowing the preservation of semen collected from these cockerels for future use and for export if necessary.

Conservation of germ plasm from domestic and endangered species via sperm cryopreservation has been practiced for decades (Gill & Barbato, 2001). Chickens were the first animals to be produced from frozen sperm using glycerol as a cryoprotectant (Donoghue & Wishart, 2000), but since then there has not been considerable progress in the development of semen cryopreservation technology in the poultry industry. Currently there is little, if any, commercial use of frozen stored poultry semen because of the reduced fertility of the sperm. The poor results may also be ascribed to the poor transport of sperm in the hen reproductive tract following cryopreservation and poor general fertility (Donoghue & Wishart, 2000).

The cockerel ejaculate is generally low in volume, but highly concentrated so there is a potential of extending it with relevant diluents, at specific rates, prior to AI and storage. Cryopreservation in domestic birds has been studied comprehensively over the past years and improvement of gamete cryopreservation has been one focus of the scientific community. However, efficient methods to freeze chicken semen of different breeds have emerged in the last decade of the 20^{th} century (Blesbois, 2007). In order to make certain that maximum success is achieved, not only required proper diluents and sperm dilution rates, but also a complex knowledge of the sperm and its physiology, is vital (Purdy, 2006). There are many unique characteristics of cockerel sperm that limits its viability for AI, either fresh or post freeze/thawing. The sperm motility and fertilizing ability of cockerel sperm generally deteriorates within 1 hour after collection, if stored in vitro (Dumpala *et al*., 2006). The avian sperm head is also unique, being cylindrical and not wide in diameter (approximately 0.5 µm) and containing less cytoplasm, which makes it difficult for the cryoprotectant to be absorbed by the sperm head cell resulting in a poor survival rate during the freeze/thawing process. The tail of the sperm is quite long (90 to 100µm), about 8 times the length of the head, relatively

longer when compared to bull sperm where the tail is 50µm thus making cockerel semen more prone to cryopreservation damage during freezing (Donoghue & Wishart, 2000).

The freezing of semen is generally performed in gradual steps, to avoid injury to the delicate sperm cells (Makawi *et al.,* 2007). Semen collection is the first critical stage of AI and successful collection results in high quality semen being obtained and cryopreserved, with the maximum number of sperm being collected per ejaculation. This emphasizes that semen collection cannot be performed by anyone and proper procedures have to be followed to achieve maximum acceptable quality semen. These procedures include proper handling of the cockerel as well as the semen, as improper handling may lead to lower quality of semen and subsequently poor conception rates (Hafez & Hafez, 2000).

Not many research studies on cockerel semen cryopreservation have been carried out in Southern Africa. This is also evident from the limited literature available. The aim of this study was thus to evaluate and document the factors affecting cockerel semen cryopreservation in different breeds.

Objectives

- To characterize fresh semen parameters from different breeds of cockerels (layer and dual breeds) farmed in South Africa

- To measure the effect of Beltsville Poultry Semen Extender (BPSE) and Dimethylsulfoxide (DMSO) on the viability and reproduction efficiency following the cryopreservation of chicken semen for different South Africa cockerel breeds in terms of:

Post thaw sperm motility

Fertilizing ability post thawing/AI

Hatchability of eggs post AI

Chapter 2

Literature Review

Effect of extenders and cryoprotective agents on the post thaw viability and fertilizing ability/capacity of poultry sperm

On poultry farms genetic selection is carried out mainly on the basis of family and individual indexes. These include traits desired in a particular type of commercial production system e.g. in a specific poultry specie, continuous genetic selection towards meat or egg production decreases natural mating efficiency and semen quality, hence lower fertility levels (Lukaszewicz & Kruszynski, 2003). The conservation of germ plasm from domestic and endangered avian species is essential and to this end, sperm cryopreservation has been practiced for decades. So for example chickens were the first species reproduced using sperm cryopreserved in a buffered diluent containing glycerol (Gill & Barbato, 2001).

Fertile eggs have been obtained from hens inseminated with frozen cockerel semen, although no live chicks were produced (Blesbois, 2007). Failure in the use of cryopreserved poultry semen has previously prevented the poultry industry from directly benefiting from genetic gains to be obtained over time, and has forced the maintenance of unique alleles by the continuous transmission of unused lines. The reason for this failure being that freeze-thawing elutes proteins from the sperm surface that is important in the sperm-egg binding process, which is also why the reduction in fertility is greater than has been predicted in post-thaw cockerel sperm motility (Gill & Barbato, 2001).

2.1 Poultry breeds in Southern Africa

Fowls for Africa is a project that brings poultry production to the people in Africa, by providing the necessary extension, knowledge and resources. It achieves this goal by

providing poultry breeds adapted to the African environment, introducing primary poultry health care aspects and training people in poultry production. Training is generally provided by scientists of the Agricultural Research Council (ARC) at Irene in South Africa, to a wide range of stakeholders, such as extension officers and prospective small-scale poultry farmers (ARC, 2006).

2.1.1 Indigenous chicken breeds

The Fowls for Africa project thus promotes the unique genetic make-up of indigenous poultry breeds, by allowing them the brooding, hatching and the rearing of their own offspring, in low input production systems. The Potchefstroom Koekoek, Naked Neck, Venda and Ovambo are examples of the indigenous broiler breeds that can be kept either under these extensive or semi-intensive systems (ARC, 2006). Most of the rural households in Africa keep these indigenous chickens for meat and egg production, a source of readily available consumable protein. However, the limited production potential of the South African indigenous chicken breeds in large numbers may be attributed to their slow growth rate, poor egg production and high rearing mortalities, when compared to other exotic or hybrid chicken breeds in commercial systems (Molekwa, 2007).

2.1.2 Exotic chicken breeds

The focus of the commercial poultry industry is mainly on the efficient production of broilers and eggs under intensive husbandry systems. The New Hampshire, Black Australop, and Rhode Island Red are examples of dual-purpose exotic breeds that are currently used in intensive systems for either meat or egg production. The Ross and Cobb lines are also excellent exotic breeds for meat (broiler) production, while the Hi-line or Lohmann are the best egg producers (NAFU, Farmer Technology, 2008).

2.1.3 Indigenous and Exotic Breeds used in the Poultry Industry

2.1.3.1 Potchefstroom Koekoek

The Potchefstroom Koekoek originated by crossing of the Black Australop with the White Leghorn breed. The term Koekoek describes the colour pattern of the bird, rather than the breed. The average weight at 20 weeks of age is 2.4kg in males and 1.7kg in females and the bird reaches sexual maturity at 130 days. The feather coloring is also sex-linked, which makes it very useful in breeding programmes. If a red or black cockerel is crossed with a Koekoek hen, the sex of the offspring can be separated when the chicks are one day old, as the males have a white spot on the head and females are completely black. This dual-purpose breed is well adapted for household production, especially in the rural areas. The hen lays on average 198 eggs during her first cycle of approximately 10 months. The first chickens with the Koekoek coloring in South Africa were the Dutch Blue breed. Later the Barred Plymouth Rock breed of chickens was imported from the United States of America and also known as Koekoek breed. This breed was popular as it laid a large number of dark brown eggs. When slaughtered the hen has a very attractive deep yellow meat (ARC, 2009).

2.1.3.2 New Hampshire

This is a dual purpose chicken breed that originated in the United States of America and is classified as a heavy breeder, with the cockerel weighing up to 3.9kg, and the hen 3kg. This breed represents a specialized selection out of the Rhode Island Red breed and was selected for its good carcass qualities, rapid growth, fast feathering, early maturing trait and vigor. The hen possesses a fair egg laying ability. The New Hampshire has a single and medium to large comb size, and in females it often lops over (ARC, 2006).

2.1.3.3 Rhode Island Red

This breed also originated in the United States of America and is a dual-purpose chicken which is renowned for its high egg production ability, and its adaptability to general household production. The cockerel weighs 4kg while the hen weighs 2.5 to 3kg (Ashraf *et al.,* 2003; ARC, 2006). The Rhode Island Red generally has a better feed efficiency, compared to the White Leghorn and this feed efficiency may be attributed to a heavier egg weight and higher egg production per day. This higher production may also be due to its superior genetic potential (Ashraf *et al.,* 2003).

2.1.3.4 White Leghorn

Leghorns originated in Italy, hence why it was formally known as an Italian breed. The name leghorn originates from the City of Ligurian Sea, from where they were first shipped. Currently it is the most popular egg laying breed in the world, mostly used in commercial production systems. Hens weigh 1.8 kg and are one of the smallest standard breeds of chicken. The Leghorn comes in both single and rose comb varieties, besides the standard white colour of the leghorn. The colouring may also come in light brown, dark brown, buff, black, red, Columbian, golden duckwing, and black tailed red. The most popular variety in the world is the pure white plumage. The White Leghorn lays large white eggs and it has an excellent feed to egg conversion ratio. The White Leghorn has little tendency towards broodiness, hence its importance to commercial egg production. With its small size it is not really suitable for meat production (ARC, 2009).

2.2 Basic Anatomy and physiology of the hen's reproductive tract

The reproductive tract of the hen is suspended in the body cavity by a ligament that attaches the tract throughout its entire length to the dorsolateral part of the body cavity. In most hens

only a left oviduct is present. The oviduct is comprised of the infundibulum, which is the site of fertilization, and engulfs the ovulated ovum. The narrower part of the infundibulum is known as the chalaziferous region, and contributes in the formation of the chalazae and is one of the two known sperm storage sites in the oviduct (Hafez & Hafez, 2000).The magnum region is the longest part of the oviduct and is creamy-white in color, with thick walls. The majority of protein albumen is formed in the oviductal tissues of the magnum, and is deposited in the ovum when it reaches this region (Taylor, 2003). The albumen further constitutes up to 54% of the egg white and is secreted by the tubular glands, found in the magnum (Etches, 1996). The isthmus region of the oviduct is separated from the magnum by a narrow translucent area which does not possess any tubular glands and contributes to the formation of the egg membranes and about 80% of the time required for egg formation in the oviduct is spent in the uterus, or shell gland region (Taylor, 2003). The mucosa of the isthmus is folded into primary and secondary ridges which are aligned longitudinally and the tubular glands in this region have secretory cells which are believed to secrete the cores of the fibers that make up the shell membrane, while the secretory cells of the epithelium secrete the mantle that surrounds them (Etches, 1996). The egg spends 18 to 22h in the shell gland of the oviduct, absorbs approximately 15g water, and exchanges several electrolytes, including sodium, potassium and chlorine. This gland contains several types of secretory cells in both the epithelium and the tubular gland. Water containing electrolytes is absorbed by the egg in this region and it decreases with an increase in the rate of shell calcification (Taylor, 2003).

2.3 Sperm storage in vivo

Avian sperm can survive in the female reproductive tract and are capable of fertilizing eggs for days or weeks in many species. The occurrence of anatomical structures associated with sperm storage was first discovered by Van Dremmelen (Hatch, 1983). The female genital

tract of the hen has crypts called sperm nests, sperm glands or sperm-host glands which occur in the infundibulum and the uterovaginal junction. Sperm introduced by copulation or artificial insemination are stored in these crypts and retain their fertilizing ability for a long period of time (Koyanagi & Nishiyama, 1981). A model accounting for the mechanism of sperm storage was deduced from the behavior of motile sperm in vitro, sperm storage tubule (SST) histology, and the SST epithelial cell ultra structure. Sperm residing in the SST were considered to be immotile. It is however likely that residence depends upon the sperm moving against the current generated by the SST epithelial cells (Froman & Feltmann, 2005).

2.4 AI in poultry

AI or artificial insemination in poultry is the process of collecting semen, evaluation of the semen, extending it with appropriate extenders, for either short (24 h or less) or long-term preservation, thawing and then manually placing the semen into the sexually receptive female tract. This technique is performed to avoid the spread of venereal diseases by natural mating and to increase the dissemination of genetic material to a large number of birds. Long-term storage of cockerel semen has been reported to be achieved by using a suitable cryoprotectant and storage at -196°C (liquid nitrogen). Subsequent fertility of the cockerel semen after AI has been reported to be between 60 and 70%. During insemination, the volume of semen required is generally less than 0.1 ml, within a minimum of 100 to 200 $x10^6$ viable sperm per insemination within the hen's vagina (Gordon, 2005).

The assessment or evaluation of poultry semen can be used as an indication of the quality of the seminal characteristics of the birds and their reproductive performance. The physiology of chickens and turkeys differ from that of other farm mammals in many ways. So for example hens lay eggs and the young develops outside the body of the dam, which is made possible by the large amount of yolk within the egg. Thus the development of the chick is completely

independent of the hen. The hen also has the ability to ovulate a mature egg daily, with only a single left ovary, while mammals have two ovaries and the period between ovulation is much longer (Gordon, 2005).

2.4.1 Behaviour of sperm in the oviduct of the hen

Froman and Feltmann (2005) reported that the hen's SST are located between the vagina and shell gland of the oviduct. Previously sperm residing in the SST were considered to be immotile, however it is likely that storage depends on moving against a current, generated by the sperm storage tubule epithelial cells. Cockerel sperm are motile at a body temperature of 41˚C for an interval of days to weeks following ejaculation. How the sperm enter, survive, and exit these sperm storage tubules however is not known. Movement of sperm to the uterovaginal region is fast, however only viable sperm enter the sperm storage tubules. Current evidence suggests that the release of stored sperm is episodic, although it was first thought to be associated with oviposition. Movement of sperm through the oviduct is achieved by smooth muscle contractions and/or ciliary activity and accumulates in the mucosal folds and short tubular glands at the lower end of the infundibulum (Hafez & Hafez, 2000).

According to Hafez and Hafez (2000) the sperm in mammals spend a relatively short time in the female tract, while in chickens and the turkey sperm can spend a much longer period of time in the oviduct before fertilizing the egg yolk cell - up to 32 days in the chicken and 70 days in the turkey. Tabatabaei *et al.* (2009) stated that although the process of prolonged sperm storage is not known, it is thought to include a reversible suppression of respiration and motility of the sperm, as well as stabilization of the plasma membrane and maintenance of the acrosome.

According to Mauldin (2000), sperm are released from the sperm storage tubules to fertilize the sequentially ovulated ova at regular intervals. After release the sperm are taken to the ovum by contraction of the hen's oviduct, and sperm motility is no longer critical. Within 5 to 10 minutes after ovulation, sperm has already moved to the genital disc on the surface of the ovum.

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

2.4.2 Artificial Insemination techniques in Poultry

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

2.4.2.1 Intra-peritoneal insemination

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

2.4.2.2 Vaginal Insemination

This is the most commonly used AI procedure and two people are required for this operation. The placement of semen into the hen's reproductive tract is accomplished by inverting the cloaca of the hen to expose the opening of the vagina. The insemination pipette is inserted

and sperm cells delivered to a location near the SST. The cloaca is everted by holding the thighs of the hen between the thumb and forefinger while the body rests in the palm of the left hand (Etches, 1996). Hens have to be inseminated for two consecutive days for the first time, and thereafter once a week, if fertile eggs are required. Cockerel semen has a limited life outside the body, and must be deposited in the hen within 1h of semen collection from the cockerel, in the case of fresh semen. It is best to inseminate hens in the afternoon (14:00 and 16:00) as in the morning hens may have an egg in the oviduct, making difficult it for the sperm to swim up to the ovary. Eggs are normally fertile after the second day of insemination, and can remain fertile for 2 or more weeks (Martin, 2004). It has been shown that hens inseminated with fresh semen produce more fertile eggs than hens inseminated with frozen/thawed sperm (Blanco *et al.,* 2000). The fertility of the eggs can also be influenced by the transport of sperm. Hens that lay eggs intensively lay fewer infertile eggs and have greater duration of fertility, compared to poor layers (Lake, 1983).

2.5 Fertility and hatchability following Artificial Insemination (AI)

2.5.1 Semen quality

Saacke (1983) stated that historically, semen quality traits have been classified according to the sperm viability or morphology. The morphology of sperm is also considered to reflect the physiological status of the male for sperm production and reflects the viability of storage in the gonadal ducts. Viability, as other semen traits, can reflect the fertility status, but it also measures the human's interaction with semen as it is collected, processed and inseminated. Semen traits related to fertility e.g. sperm motility, velocity, acrosome morphology etc., may affect the penetration of the cervical mucus and are thus important for semen preservation and ultimately fertilizing capacity.

Parker and McDaniel (2002) reported that in poultry there is no real breeding soundness evaluation. Roosters are selected, based on physical characteristics, that are associated with the mature males e.g. comb and wattle size and colour, body size and shank length. However, it is very important to evaluate the semen quality, as it predicts the fertility of an individual male. Fertility and hatchability according to Tabatabaei *et al.* (2009) depends on genetic, physiological, social and environmental factors. These are interrelated heritable traits, which may vary between breeds. Sperm numbers, type of hens (broiler or layer) and age may affect the in vivo storage of sperm, and subsequently the fertility of the eggs.

The conditions which cause low sperm numbers or single sperm activity at the site of fertilization can cause a reduction in the actual number of chicks being hatched. Low fertility and early embryonic mortality may be the result of low sperm activity or single activity. This condition is normally associated with older breeder hens or any flock experiencing infrequent mating activity (Bramwell, 2002).

The fertility and the number of stored sperm in the hen's oviduct increases with an increasing number of viable and motile sperm inseminated. It has been estimated that a weekly AI dose of 50 to 300 $x10^6$ sperm per insemination is required to maximize fertility. Lower insemination doses could be used if the semen quality is high (Parker *et al.,* 2002). Storage of eggs can however also affect the fertility and hatchability. So for instance if eggs are stored for more than a week there is known to be an increase in the occurrence of embryonic abnormalities and mortalities due to the degradation of viscosity of the egg albumen. In addition these eggs show a reduced hatchability and an increased incubation time required to hatch, while also causing deterioration in the growth rate of the chicks after hatching (Petek & Dikmen, 2006). Temperature, humidity, gaseous environment, and the orientation and positional changes of the eggs affect hatchability and chick quality during incubation. Long

storage prolongs the incubation time, which can also have a negative effect on the chick survival. Short time storage of eggs between laying and incubation has been shown to have the highest hatching potential (Reis & Soares, 1997)

Table 2.1 Factors reported to influence hatching success of chickens during the production period and the possible sources (Christensen, 2001).

Stage	Possible Sources	Possible Mechanisms
Development at oviposition	Genetics Age of hens Time of oviposition Egg weight and quality Body temperature	Genetic Difference Ovulation intervals Time in oviduct Maternal investment pH, albumen, $C0_2$, embryo metabolism, chemical/physical properties
Egg storage development	Genetics Nest type Collection rate Heat conductance value of egg components Egg quality Time in storage Temperature Humidity	Type of egg Cooling rate of eggs Cooling rate of eggs Cooling rate of eggs, escape of $C0_2$, Eggshell porosity pH, $C0_2$ and chemicals Chemical/physical properties Unknown
Incubation development	Temperature Humidity Ventilation Turning Genetics Semen storage Age of hen Egg storage	Chemical/physical properties Chemical/physical properties Chemical/physical properties Adherence to membranes, angiogenesis Apoptosis, DNA regulation Unknown Embryo growth rates Embryo growth rates, pH, $C0_2$

2.5.2 Factors associated with the insemination procedure

- Number of sperm inseminated into the oviduct

A high level of fertility throughout the breeding season can be maintained by a minimal number of high quality sperm being inseminated at regular intervals. In general one insemination per week with 80 to 100 x 10^6 fresh sperm will be sufficient to maintain high fertility rates. Inseminating birds with more than 100 x 10^6 fresh sperm per insemination has been shown to make no difference, when compared to 80 to 100 x 10^6 million sperm per insemination. The transparent fluid may reduce the density of the semen dose and it would appear as if the insemination of 0.025ml to 0.05ml semen twice weekly or every 4 or 5 days is acceptable to maintain constant fertility in the hen. The frequency and number of sperm inseminated may be increased from the middle to the end of the breeding period to overcome a decrease in fertility at that specific time. However, it will not succeed if the decrease is irregular, e.g. it may be caused by a reduction in the number of sperm in a fixed semen volume, or due to either improper application of the technique, age of the male or a seasonal decline in the semen quality of the cockerel. This change in reproductive capacity of the male occurs simultaneously with a change in the fertility of the female (Lake, 1983; Surai & Wishart, 1996; Parker *et al.,* 2002; Tabatabaei *et al.,* 2009)

- The deposition of semen and timing of insemination

During AI, the vagina must be inverted before the semen can be deposited in the oviduct, ensuring that the semen is deposited as close to the sperm storage glands in the proximal area of the vagina, as possible. Timing of insemination is very imported to obtain high fertility rates and inseminations must be performed when no hard shelled egg is likely to be present (ostruction) in the uterus. Hens must also be handled with care during capturing before AI

and released gently after insemination, otherwise semen may be regurgitated from the vagina. Any degree of stress caused to the birds may interfere with the transport of the sperm, and have a consequent effect on the fertilization rate (Lake, 1983; Donoghue & Wishart, 2000; Obidi *et al.,* 2008,).

- The reproductive physiology of the hen and the activity of sperm in the oviduct

Fertility levels after insemination may be influenced by the effect of the oviductal environment on the transport of the sperm and the retention in their fertilizing capacity. Any change in the hen's oviduct, either environmental or physiological may lead to unexplicable fertility problems. A decline in the fertility of hens exposed to high environmental temperatures, could partly be due to a defective oviductal environment, affecting the metabolic activity of the sperm. The start of the decline in fertility in the hen differs with age, season (according to egg productivity) and with the type of bird, e.g. in broilers fertility declines sooner than in layers (Lake, 1983; Donoghue & Wishart, 2000,).

- Immunity against sperm

Fertility levels in fowls and turkeys bred by AI may be affected by the hens generating antibodies against the sperm, causing the sperm to be ineffective. This may result in an occasional decrease in fertility during the course of the breeding period. However the evidence is controversial, as the inexplicable variation in fertility levels could be due to various other factors, e.g. disease, environment, nutrition, a decrease in semen quality or a faulty artificial insemination technique (Lake, 1983).

2.6 Anatomy of the cockerel's reproductive tract

The primary sex organs of the cockerel are the testes, with their main function being the production of sperm and the male sex hormone, testosterone. Both testes are functional in the

male when sexual maturity is attained. The size of the two testes may differ, the left testis usually being 0.5-3g heavier than the right testis (Etches, 1996). The gross weight of the paired testes is on average 25g, and the sperm produced per gram of testicular parenchyma is approximately 100 x 10^6, with a daily sperm production of 2.5 x 10^9sperm/ml. An accurate method of determining the quantity of sperm that a bird can produce is generally by measuring the circumference of the testes, e.g. the larger the size of the testes, the greater the sperm production (Senger, 2003). The testes in the cockerel are located in the centre of the body cavity, and spermatogenesis occurs at body temperature (41°C), as opposed to the mammalian scrotal temperature of 24 to 26°C (Tuncer, *et al.,* 2006).

In certain mammals the testes are located outside the body, while in the cockerel the testes, as indicated, are located inside the body cavity. The cockerel's reproductive tract is comprised of a duct system, with a paired epididymis and vasa deferentia. Seminal vesicles, a Cowper's gland, prostate gland and a penis are absent, other than in mammals. Before copulation in the cockerel, the vas deferens increases in diameter allowing semen to be stored in the bulbous region. Semen is then released from the vasa deferentia during sexual stimulation (Perry, 1960).

In most domestic animals the production of semen varies according to the season of the year. During the natural breeding season there is a high production of quality semen, compared to the other times of the year. The cockerel may have normal, degenerated partially degenerated sperm tubules, which would result in the production of normal or abnormal sperm. There are many factors that can affect these seminiferous tubules e.g. seasonal influences and dietary deficiencies which may cause a reversible degeneration of the tubules and thus affect spermatogenesis and hence poor sperm production (Anderson, 2001).

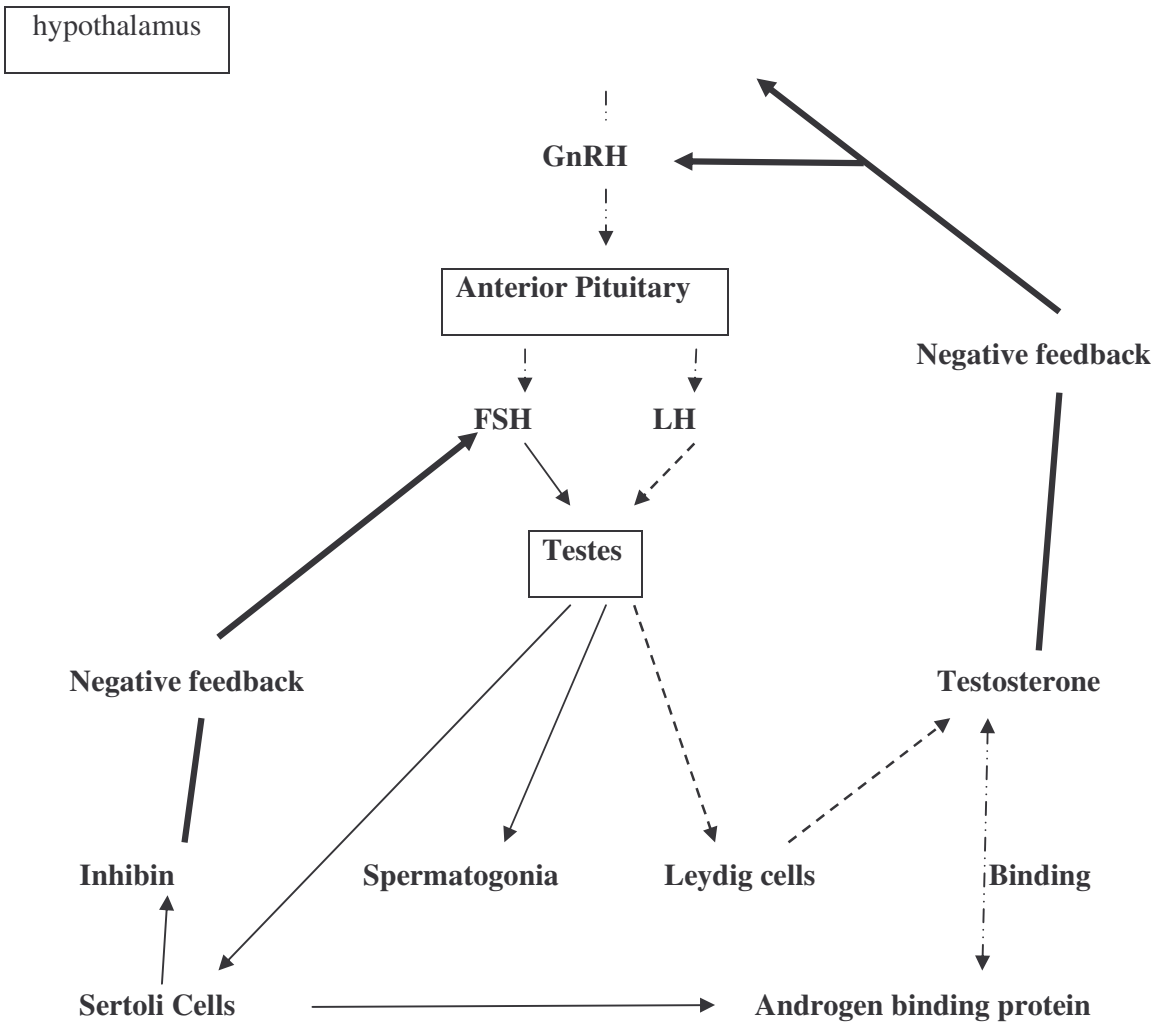
2.6.1 Body weight of the cockerel

The body weight of the cockerel is important when selecting for breeding flock performance. A significant negative correlation has been established between the growth rate or body weight and the reproductive performance in males (Harris *et al.,* 1980; Lukaszewicz & Kruszynski, 2003). It is crucial for the males to reach a minimum body weight typical for a given breed, strain or type, before being used for breeding (Lisowski & Bednarczyk, 2005). The number of sperm per ejaculate and body weight has been positively correlated and it may be concluded that body weight and length of the shank, comb and wattle are good predictors of semen attributes in cockerels. This is contrary to the reported negative effect of body weight on semen production (Wilson *et al.,* 1979; Galal, 2007). Harris *et al.* (1980) however recorded a positive correlation between body weight and semen volume when cockerels were 48 weeks of age. However, this relationship was not observed when the males were 30 or 40 weeks of age. As would be expected, the body weight of the cockerels increased with an increase in age and the male body weight noticeably influenced the percentage of reproducing males with age. The overall mean age of peak percentage of males regarding semen production, was 44 weeks of age.

2.7 Physiology of cockerel reproduction

Production of sperm is initiated by adequate secretion of GnRH from the hypothalamus, the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the anterior lobe of the pituitary and the secretion of the gonadal steroids (testosterone and estrogen). LH acts on the Leydig cells within the testes to stimulate the production of progesterone, which is converted to the male sex hormone testosterone. Testosterone within the seminiferous tubules is essential for spermatogenesis, while the Leydig cells become unresponsive sustaining high levels of LH (Senger, 2003).

Figure 2.1 The interrelationship of the endocrine hormones regulating reproduction in the male (Beardon *et al.,* 2004)



Key of the diagram

Action of FSH

Action of LH

Negative feedback

Positive feedback

The testes are surrounded by a layer of connective tissue containing the seminiferous tubules and Leydig cells. Several androgens are produced in the interstitial cells of the testes, but the major hormone in the blood, is testosterone. Testosterone is essential for the development of the secondary sex characteristics and for normal mating behaviour in the males. It is also necessary for the functioning of the accessory glands, sperm production and the maintenance of the male duct system. This hormone also aids in spermatocytogenesis, the transport of sperm and deposition of sperm in the female reproductive tract (Beardon *et al.,* 2004). As the cockerel reaches maturity, the production of testosterone is stimulated by the increasing concentration of circulating gonadotrophins (Etches, 1996). The major gonadotrophins involved are FSH and LH, which are also called the interstitial cell stimulating hormone (ICSH) in males. Both of the gonadotrophic hormones are secreted by the anterior pituitary (Salisbury *et al.,* 1978). FSH as such, acts on the germinal cells in the seminiferous tubules of the testes and supports spermatogenesis to the secondary spermatocytes stage. LH stimulates the Leydig cells to produce testosterone and other androgens (Hafez & Hafez, 2000).

2.7.1 Functions of the accessory sex glands in the cockerel

The prostate, vesicular and bulbo-urethral glands are the accessory glands present in most domestic animals, with their main function being the production of secretions that aid in the sperm transport and contain specific chemical agents. So for example fructose and citric acid are components of the seminal vesicle secretions in domestic animals, with citric acid being found only in the stallion's seminal vesicles. The prostate is the only accessory gland common to all mammals, while the epididymis and the vas deferens are the only accessory organs present in poultry (Hafez & Hafez, 2000). Domestic birds or more specific cockerels have no secondary sex glands. Therefore, the seminal fluid is derived entirely from the testes and the underlying ducts. Additionally when semen is collected from the cockerel by

abdominal massage, there are lymphatic exudates from the phallic folds which contribute to the ejaculate. It is not clear if this lymphatic fluid is a normal avian semen component, as the composition of semen during natural mating in birds has received little attention (Etches, 1996).

2.8 The process of spermatogenesis

Spermatogenesis is the process of division and differentiation by which sperm are produced in the seminiferous tubules of the testes and consists of two phases, namely spermatocytogenesis and spermiogenesis (Gordon, 2005).

2.8.1 Spermatocytogenesis

Cross sections of the seminiferous epithelium form well defined cellular associations that undergo cyclic changes. The numbers of distinct cellular stages differ in different types of domestic animals, e.g. 14 distinct stages are identifiable in some species, whereas only 6 stages are identified in the human and 12 in the bull. The time needed to complete the cycle of the seminiferous epithelium, varies between domestic species. Four to 5 epithelial cycles, depending on the species, are required before the type A spermatogonia from the first cycle have completed the metamorphosis of spermiogenesis. However the rate of spermatogenesis is uniform within a species, and is estimated to be 12.8 days in the cockerel (Hafez & Hafez, 2000).

The seminiferous tubules in poultry are arranged as a network of interconnected ducts that empty into the rete testis and their periphery is lined with spermatogonia, which are normally considered to be involved in the first stage of spermatogenesis (Etches, 1996). There are two types of the cells in the seminiferous tubules, namely the germ cells or spermatogonia and Sertoli cells, which are somatic cells. Sertoli cells extend from the basement to the lumen and

play a role in supporting spermatogenesis (Beardon *et al.,* 2004). The spermatogonia are specialized diploid cells found in the basal compartment of the seminiferous epithelial and are produced continuously by mitotic division to yield subsequent generations of spermatogonia and spermatocytes which enter the first meiotic division. Differentiation of spermatogonia into haploid spermatocytes requires the participation of the Sertoli cells to support sperm production, which also provide the micro-environment in which differentiation can take place, and also nourishes the developing sperm (Tuncer *et al.*, 2008).

2.8.2 Spermiogenesis

According to Gordon (2005), spermiogenesis is a metamorphic process in which no cell division is involved and a string of events result in the formation of the sperm tail. Alteration in the sperm morphology can be seen in the nuclear proteins, cellular size, cellular shape and the position of the acrosomal granules and localization of the centrioles. The number of sperm produced is dependent on the number of Sertoli cells and Leydig cells present. The Golgi apparatus is one of the cell organelles, located near the sperm nucleus and which give rise to the sub-cellular organelle known as the acrosome. The acrosome develops and forms a cap over the anterior portion of the nucleus and spreads until it covers two-thirds of the anterior nucleus (Senger, 2003). During the maturation phase, the spermatids are completely differentiated with the final formation of the flagella (principal and end-piece), assembly of mitochondria (mid-piece), the neck piece and complete condensation and shaping of the nucleus (Beardon *et al.,* 2004).

Table 2.2 Characteristics and the mean chemical components of semen in the domestic cockerel (Hafez & Hafez, 2000).

Characteristics and Components	Cockerel
Ejaculate volume (ml)	0.2-0.5
Sperm concentration in ($x10^6$/ml)	3000-7000
Sperm/ejaculate (billion)($x10^9$)	0.06-3.5
Motile sperm (%)	60-80
Morphologically normal sperm (%)	85-90
Protein (g/100 ml)	1.8-2.8
pH	7.2-7.6
Fructose (mg/100ml)	4
Sorbitol (mg/100ml)	0-10
Inositol (mg/100ml)	16-20
Glyceryl phosphoryl choline (GPC) (mg/100ml)	0-40
Ergothioneine (mg/100ml)	0-2
Sodium (mg/100ml)	352
Potassium (mg/100ml)	61
Calcium (mg/100ml)	10
Magnesium (mg/100ml)	14
Chloride (mg/100ml)	147

2.8.4 Cockerel semen composition

In the male, semen is composed of sperm and seminal plasma secreted by the epididymis and vas deferens. The sperm are produced in the testes, and in the case of the avian species the seminal fluid is also produced in the testes. All these secretions in the testes are controlled by the endocrine hormones carried to them in the blood stream. The pituitary FSH and LH regulates the testes, which in turn produce testosterone, which controls the testicular development and secretions (Hafez, 1974).

2.9 Factors affecting semen production

There are inherent variations in semen production between different species of poultry and between individuals within strains and breeds (Lake, 1983). Other than in the mammal, cockerel sperm is generally immotile before ejaculation (Hafez & Hafez, 2000). According to Anderson (2001) there are many factors that may influence the production of semen and a thorough knowledge of the physiology of cockerel reproduction is essential to enable an understanding of male fertility. There are also many external and internal factors that may affect the male and may influence the production of semen. The reproductive functions in the male are endocrine controlled by the pituitary, testes and to a certain extent, external factors.

The certain external factors affecting reproductive efficiency in the cockerel can be grouped into two categories, firstly, the direct influence of the diet, management, and the normal physiological processes that regulate the activity of spermatogenesis and secondly factors that influence the degree to which the male will respond to the massage technique during semen collection (Maule, 1962).

2.9.1 Ambient Temperature

Direct climatic factors acting on the birds include high ambient temperature and relative humidity, resulting in severe heat stress. Heat stress can be one of the main limitations in poultry production and reproduction, more especially in hot areas. Elevated environmental temperatures pose a threat to the general well-being of the cockerels. The increase in the body temperature without a rapid compensation of heat loss, resulting from a prolonged exposure to environmental temperature, may cause a change in the body temperature of the cockerel body, leading to a significant impairment of semen production and reproduction. The intensity and duration of heat stress combined with relative humidity may also affect the behavioral, hormonal and physiology of the cockerel. Such detrimental effects limit reproduction characteristics of the males thus inhibiting spermatogenesis and a decrease in the secretion of gonadotrophins (Bah *et al*., 2001; Ayo & Sinkalu, 2007; Obidi *et al*., 2008; Oguntunji *et al.,* 2008). Body temperature increase, sperm metabolism, sperm motility and sperm quality are generally lower in heat-stressed cockerels. Although research concerning hyperthermia on semen characteristics is lacking, several researchers have found that sperm can function at normal body temperature (Karaca *et al.,* 2002). Froman and Feltmann (2005) found sperm to be motile at a body temperature of 41˚C, and decline with time after ejaculation. Heat stress may be evaluated by measuring the rectal temperature which is the true reflection of the internal body temperature (Ayo & Sinkalu, 2007).

2.9.2 Photoperiod or daylight length

Most domestic birds are seasonal breeders and in most birds, photoperiod stimulates spermatogenesis, especially semen production in e.g. the White Leghorn. The duration and intensity of photoperiod may have an effect on the conditioning of the chickens for reproduction (Anderson, 2001). Short days do not stimulate gonadotrophin secretion, as they

do not illuminate the photosensitive phase. However, long days illuminate the photosensitive phase and therefore the gonadotrophin LH is secreted. Photo-schedules in poultry production are currently being practiced, and designed to maximize the yield of semen for a prolonged period, by delaying the onset of photorefractoriness. In addition, the generation interval can be reduced when photostimulation is practiced at an earlier age (Etches, 1996). Time of the day for the collection of semen also affects the quality and quantity of cockerel semen. Generally semen production is higher in the morning and in the afternoon, when it is cooler (Peters *et al*., 2008). The breed of poultry also contributes to a difference in semen production capability. The production of semen also differs within seasons, being regulated primarily by daylight length or photoperiod. The chicken breeding season generally starts in spring when the daylight length is long and terminates when the daylight length is even longer, due to the effect of the refractoriness (delayed response to long day length), of the pituitary gland (Gordon, 2005). According to Hafez and Hafez (2000) the onset of reproduction occurs when light, acting through photoreceptors in the brain, provides neural signals which the bird's reproductive endocrine system perceives as a change in daylight length, sufficient to initiate reproduction. The neural signals with time fail to maintain gonadotrophin secretion, despite continued light stimulation. Refractoriness is characterized by a gradual decline in LH, which causes a gradual decline in the egg production until the pituitary can no longer secrete sufficient LH. The mechanism of this ovarian regression appears to reside in the hypothalamus where luteinizing hormone releasing hormone (LHRH) is synthesized.

2.9.3 Nutrition

Feed restriction causes stress in cockerels, while low water intake induces the males to lose body weight. This disruption can lead to a permanent non-functional testis and a reduced reproductive performance in the mature cockerel. The nutrient requirements of males have

generally received less attention, and it is a common practice that cockerels are given the same diets that have been formulated for the hens. This affects the males in that they often suffer from chronic gout due to high amounts of calcium and protein intake that exceeds their metabolic requirements. The diet also affects the production of semen, in that the production of semen is decreased, although the semen characteristics are not affected. Males consuming less quantities of protein in the diet ejaculate more frequently, however, their lifetime sperm output far exceeds that of males consuming higher amounts of protein (Perry, 1960; Etches, 1996).

2.10 Techniques of semen collection

Care must be taken before and during semen collection in poultry to avoid any semen contamination by the collecting equipment, blood and the cloacal products, to maximize semen quality and quantity (Lukaszewicz, 2002). Cockerels need to be trained before semen can be collected for use in AI. Briefly the cockerel must be taken gently from the cage (minimum stress) and manipulated immediately. Once the initial excitement is missed, the reflex massage is difficult to elicit, together with ejaculation. The males have the tendency of defaecating and urinating when stimulated for the first time, until they are adapted to the ritual of semen collection. The cleanliness, quality and quantity of semen ejaculated may depend on the pressure exerted on the ejaculatory ducts (Maule, 1962).

2.10.1 The massage technique of semen collection

This technique was first described by Burrows and Quinn (1937). By using this method, the cockerel is massaged in the dorsolateral lumbo-sacral region or the abdomen and the tail is pushed forward over the males back. This massage causes the copulatory organ to become erect and rapidly secrete semen from the ejaculatory ducts. The extent of erection is

dependent and varies with each individual cockerel. This method needs the assistance of one or two people (Cole & Cupps, 1977).

2.11 Macroscopic evaluation of semen

2.11.1 General Evaluation

The evaluation of poultry semen is important for AI as it does not only involve the collection of semen, but also the quality and many other aspects are to be considered prior to use, for example contamination density of the ejaculate and the viability (motility) of the sperm. There are many parameters that can be used to evaluate the general quality of cockerel semen and estimate the extent to which semen can be extended, e.g. ejaculate volume, semen concentration, and total number of sperm, sperm motility and morphology. Semen has to be collected by a trained technician and these technicians have to be clean in order to avoid semen contamination by e.g. faeces, foreign material, etc.

2.11.2 Semen colour

The colour of semen is generally an indication of the density of the ejaculate. The semen of the domestic fowl varies from a dense opaque suspension to a watery fluid secreted by various reproductive glands, from a relative high sperm density or degrees of clear to milky white, with declining sperm numbers (Sexton, 1980; Peters *et al*., 2008). Colour could also serves as an indication of contamination by e.g. faeces or urine and thus become brown or green in colour (Lake, 1983). Sometimes flakes of blood may be present, which may be a result of excessive force being used during the collection process or injury. Semen samples that are contaminated by faeces do not have to be discarded, but diluted with antibiotics e.g. penicillin and dihydrostreptomycin or neomycin to reduce the loss of sperm. This however is

not recommended. Antibiotics can also increase fertility when used as a diluent in semen (Sexton, 1980; Bearden *et al*., 2004).

2.11.3 Cockerel ejaculate volume

The colour of semen may depend on the specie of bird used, but generally semen should be creamy which indicates a high sperm concentration (Cole & Cupps, 1977). The cockerel produces between 0.1 ml and 1.5 ml per ejaculation, with 0.6 ml being the average ejaculate volume recorded. Different cockerels of the same species often produce different volumes of semen at different times (Anderson, 2001). The average volume ejaculated using the abdominal massage technique is approximately 0.25ml and contains on average 5000 $x10^6$sperm/ml (Gordon, 2005). Bah *et al.* (2001) found the mean semen volume to be 0.28 ± 0.14ml. However, the recorded semen volume was found to range between 0.37 ± 0.02 and 0.73 ± 0.01 ml (Peters *et al.,* 2008; Tuncer *et al.,* 2008). It is important to realize that semen volume and sperm concentration (volume multiplied by the concentration) will determine the total number of sperm collected per ejaculation. This could facilitate the determination of the number of insemination doses that can be prepared (Senger, 2003).

2.11.3.1 Factors that affect cockerel ejaculate volume

The quantity of semen collected by the massage procedure is dependent upon the male species, breed, age, nutrition, frequency and technique of semen collection.

2.11.3.1.1 Species and breed of the cockerel

The active reproductive period influences the volume of semen produced. It has been reported that ejaculate volume and sperm concentration are dependant on the strain and breed of the cockerel e.g. the Naked Neck and Frizzle genotypes produced higher ejaculates, compared to the general feathered breeds of cockerels. Researchers have recommended the

use of comb length and wattle length as good indicators for the selection of quantitative traits in cockerels. Larger combs may reliably indicate cockerels with greater semen production, higher androgens levels or increased mating activity (Kotlowska, 2005; Zahraddeen *et al.,* 2005; Nwachukwu *et al.,* 2006; Galal *et al.,* 2007).

2.11.3.1.2 Nutrition

The ejaculate volume, sperm density, and fertilizing capacity of cockerel semen can be reduced by restricted feed intake. However, a reduced protein level of as low as 6.9% has shown no adverse effect on fertility in males. The body weight of breeder cockerels have to be well managed in the attainment of sexual maturity of the cockerels to coincide with that of the hens. Too heavy males tend to be over-fleshed and generally have reduced persistency in semen production. Overfed males also show a have reduced fertility, compared to cockerels fed to meet a target body weight. However, underfed cockerels recorded a reduced semen volume and low fertility (Parker & Arscott, 1963; Das, 2002; Renema *et al*., 2007).

2.11.3.1.3 Age, frequency and technique of semen collection

In poultry species, quality parameters such as semen volume, semen concentration and sperm motility changes with the age of the male, leading to a progressive decline in fertility. Semen concentration appears to be the seminal trait that is commonly affected by frequency of ejaculation, as semen concentration declines progressively with an increase in the ejaculate frequency. A report has shown the frequency of collection of semen and age of the cockerels and the biochemical parameters of the semen to be more affected by age than the ejaculate volume. The changes in semen quantity and quality may be related to an increasing age of the cockerel. It has been reported that in boars, too frequent collection of semen cause temporary loss of fertility (Hafez & Hafez, 2000; Kotlowska *et al*., 2005; Tuncer *et al.,* 2006).

2.11.4 Semen pH

The semen pH varies slightly between different breeds and bird species. The optimum semen pH ranges between 7.0 and 7.4. Sperm motility is generally high between a pH of 7.0 and 7.4 (slightly alkaline) and also increases the fertilizing ability, compared to a pH of 6.4 (acidic), which is not suitable for semen preservation, as it may cause damage to the plasma membrane of the sperm cell (Latif *et al*., 2005). Contrary, Donoghue and Wishart (2000) and Siudzinska and Lukaszewicz (2008b), reported several trials that indicate that chicken sperm can tolerate a pH range of 6.0 to 8.0. Peters *et al.* (2008) also found the semen pH of the cockerel to be slightly alkaline, with a mean of 7.01 ± 0.01, while Tuncer *et al,* (2008), and Bah *et al.* (2001) recorded a semen pH ranging between 7.54 ± 0.04 to 7.80 ± 0.03. This variation in semen pH may be due to many factors. The pH, especially that of ejaculated semen is dependant on several secretions involved. Poor quality semen generally contains large amounts of fluid from the accessory glands, which increases the semen pH. The pH of semen is likely to decrease as the time between collection and measurement increases, and the semen collection tubes are narrow in shape causing sperm to break down fructose in the semen to lactic acid under anaerobic conditions. Semen samples that contain many dead sperm may evolve ammonia, which will also increase the pH (Salisbury *et al.,* 1978).

2.12 Microscopic evaluation of cockerel semen

2.12.1 Semen concentration determination

Sperm cell concentration is defined as the number of cells per ml ejaculate and normally predicts the number of breeding units that can be inseminated (Cole & Cupps, 1977). The density of the semen samples in the cockerel range from less than 800 x 10^3 to over 6 x 10^6 sperm/ml (Anderson, 2001). It is therefore important to record the concentration of each

ejaculate of the male. Lower sperm concentrations could be indicative of a serious problem in the fertility of the cockerel. This problem may be due to disease or insufficient stimulation of the animal before collection. Normally semen concentrations are not related to infertility, however ejaculates containing less than 500 million cells per ml have been associated with low fertility rates (Bearden *et al*., 2004). Tuncer *et al.* (2008) recorded cockerel sperm concentrations of 2.42 ± 0.02 $x10^9$sperm/ml, while other researchers quoted sperm concentrations of 3.53 ± 1.00 $x10^9$sperm/ml, 2.20$x10^9$sperm/ml, 1.878 ± 0.2$x10^9$sperm/ml for White Leghorn cockerels and 3.32 $x10^9$sperm/ml, and 3.347 $x10^9$sperm/ml for the New Hampshire breeds (Tuncer *et al.,* 2006; Peters *et al.*, 2008; Tuncer *et al.,* 2008,). Similarly Bah *et al*. (2001) reported a sperm concentration of 2.26±1.08 x 10^9 sperm/ml for local indigenous cockerels in Nigeria.

The hemacytometer is normally used for the counting of red blood cells, however it can also be used to determine semen concentration. The hemacytometer has two counting chambers which are 0.1mm in depth and a ruled grid at the bottom of each chamber that is 1.0 mm square with two dilution pipettes. The size of the grid determines the number of sperm that are to be counted for a specific semen dilution. The dilution rate of the semen samples depend on the species e.g. 1: 200 is normally used for bull and 1:400 for ram semen. Counting can be accomplished by using a diluent that immobilises the sperm. The method of using the hemacytometer is however very time consuming, when used to determine sperm concentration. It needs accuracy when counting, and is subject to considerable error. The photo-electric colorimeter may also be used for semen concentration determinations in commercial semen producing operations and is a convenient and fast method. This method relies on the density of the semen sample (Bearden *et al*., 2004).

Currently the electronic particle counter is the best and most accurate method being used to determine the sperm concentration, when compared to the hemacytometer and the photo-electric colorimeter techniques. It is used to determine the number of the sperm cells in an ejaculate and is adjusted according to the particle size so that each and every particle or sperm cell is counted (Cole & Cupps, 1977). Counting of sperm in the chambers differs with the species of animals, with bulls and rams the corner and center squares are counted, while in boars and stallions 25 squares may be counted. In the cases where 5 squares are counted, the total number of sperm counted is multiplied by 5. The formula used for calculating sperm concentration is: No. of sperm/ml = No. of sperm in $0.1mm^3$ x 10 x dilution rate x 1000 (ml = cubic centimeter or ml) (Bearden *et al*., 2004).

2.12.2 Sperm Motility

Sperm motility assessment is indicative of the viability of sperm and the quality of the semen sample. Evaluation of sperm motility is conducted with raw and extended semen, and generally analyzed under the light microscope (x10 magnification). Evaluation of raw semen gives the performance of the sperm in its own accessory gland fluid, which is often hindered when higher sperm concentrations make it difficult to distinguish individual sperm motility patterns (see Table 2.3). Hence an aliquot of semen is usually extended prior to evaluation (Hafez & Hafez, 2000).

There are however several factors affecting sperm motility following semen dilution. Research has shown avian sperm motility to be dependant on the amount of oxygen and Ca^{++} ions present (Parker & McDaniel, 2006). It is also advisable to evaluate sperm motility before and after preservation, as it determines the end viability and ultimately the fertilization rate following AI. Cockerel semen is normally diluted in the ratio of 1:20, using the same media that is used for cryopreservation. There are many ways of evaluating sperm motility e.g. a

computer assisted sperm analyzer (CASA) system. This system is used to evaluate sperm motility and consists of a costly phase contrast microscope with a warm stage (37 °C), equipped with sperm class analyser software (Yeste *et al.,* 2008).

Generally microscopic sperm motility assessment is subjective, as it includes an estimation of the viable sperm. Tuncer *et al*. (2006) determined the sperm motility for Denizli cockerels and found it to be on average 72.3 ± 0.08%. Researchers have also found the sperm motility for the White Leghorn breed to be 83.2 ± 0.6%, and 77.6 ± 0.2% for the New Hampshire (Chalah *et al.,* 1999; Tuncer *et al*., 2008). The most common method of analyzing sperm motility is with the use of the light microscope using raw and extended semen samples. Sperm are very sensitive and susceptible to environmental factors such as temperature, and care should be taken to protect the sperm against temperature shock, prior to evaluation. For evaluation, a glass slide with a droplet of extended semen can be used and observed under a microscopic, with a built in warm stage and phase-contrast optics. A magnification of x200 to x400 is generally used (Hafez & Hafez, 2000).

Parameters used to evaluate sperm motility include the following (Hafez & Hafez, 2000):

- Percentage of sperm which are motile (normal is 70 to 90% motile sperm),
- Percentage of progressively motile sperm,
- Sperm velocity (based on an arbitrary scale of 0-4 with 0 being stationary and 4 fast moving).
- Longevity of sperm motility in raw semen at room temperature (20 to 25°C) and in extended semen at room temperature or a refrigerated temperature of 4 to 6°C.

Table 2.3 Motility patterns of mammal sperm from sub-fertile or infertile males (Hafez and Hafez, 2000)

Pattern of sperm motility and morphology	Sperm tail	Sperm head	Sperm movements and progression
Vibratory circular	Slow or rapid quivering from side to side; vibrations of various types and frequency bent in curved shape; immotile.	Immotile or vibrating in one place	Motility without progression; perpendicular, oblique, or horizontal clockwise or counterclockwise motion
Darting	Vibration with high velocity	Irregular; propelling; no rotation	Minimal and erratic; wandering path
Rotating	Undulations of small amplitude pass down tail	Whole sperm rotates around its axis; periodic "flashing" effect	Rapid forward progress in a straight line
Asymmetric head and /or flagella	Amplitude of tail wave is asymmetric at both sides	Irregular; propelling; usually no rotation	Circular orbits if rotational motile is absent
Sperm with cytoplasmic droplet	Amplitude of tail is unequal; rapid variations	Irregular; often rocking; seldom rotational	Perpendicular, oblique, seldom progressive
Agglutinated sperm	Decreasing, vibrating motion; slow, vibrating motion	Slow; irregular propelling; rocking	Depends upon the type of agglutination

2.12.3 Speed of Sperm

The rate of sperm motility is the speed at which the sperm travel. This speed is generally measured using electronic equipment. This equipment is however expensive to use in commercial semen processing operations and sperm motility is generally visually assessed subjectively on a scale of 1 to 5. A rate of 21mm per minute is regarded as the normal speed of an individual sperm cell, but in semen with excellent motility sperm have been shown on average to reach a speed of 15mm per minute (Bearden *et al.*, 2004) The sperm swimming speed is an important determinant of male fertility and the size of the sperm determines the speed of the sperm. Long and medium sized sperm swim faster than short sperm, and are more likely to fertilize the ova. The behavior of the sperm cell within the hen's vagina constitutes a critical determinant of fertility in the hen. The motile sperm ascend in the vagina, enter the SST, and upon their release pass rapidly to the infundibulum where fertilization takes place (Froman *et al*., 1999; Parker *et al.,* 2006; Gomedindio & Roldan, 2008; Kleven *et al.,* 2009).

2.13 Sperm Morphology

2.13.1 Microscopic Evaluation

Normally the sperm cell consists of a head, mid-piece and tail portion. The head contains the nucleus, containing the genetic material, which is the sire's genetic contribution to the offspring. The postnuclear cap which covers the posterior part of the nucleus and acrosome which covers the anterior part of the nucleus, both protect the nucleus. If the acrosome is malformed or damaged the sperm cell will not be able to fertilize the ova by penetrating the zona pellucida. Acrosome, sperm head, middle piece and tail deformations in fresh cockerel ejaculates have been recorded to be 0.62 ± 0.04%, 1.34 ± 0.05%, 2.47 ± 0.05%, and 2.89 ±

0.08% respectively (Tuncer *et al.,* 2006). While Tuncer *et al.* (2008) also recorded values of 0.39 ± 0.03%, 1.06 ± 0.03%, 2.32 ± 0.05%, and 2.53 ± 0.04% respectively.

Blesbois (2007), described an eosin-nigrosin stain technique to assess the morphology of cockerel semen. Semen was mixed with 1.6% eosin and 6% nigrosin, diluted in Beltsville Poultry Semen Extender (BPSE) (20 µm diluted semen in 2ml stain solution) and the diluted semen incubated for 2 min before being spread on a microscope slide. The stain was dried and observed under a light microscope (x1000 magnification). Sperm morphology can serve as an indicator of semen quality and short-comings in the male. The success of this evaluation technique depends on how the stain was prepared and used, while other more advanced laboratories use a computer analysis (CASA) system for sperm evaluation. Eosin-nigrosin is a die commonly used in laboratories to determine abnormalities and smears are made, immersed in oil and observed under the light microscope. Viable, non-viable, properly formed, live, and damaged sperm can be determined using this evaluation (Lukaszewicz *et al*., 2008).

The sperm morphology of poultry semen differs from that of mammals. However a difference also exists between domestic birds, even though the shape and size of the sperm cell are similar. In poultry the sperm cell is surrounded by the cytoplasmic membrane and the acrosome has an inner spine surrounded by a conical-shaped cap. The head of the sperm contains the nuclear material of the gamete, while the mid-piece consists of the cylindric centrioles surrounded by a sheath of mitochondria (Hafez, 1974). The mid-piece of cockerel sperm is considerably longer, compared to other species, approximately one quarter longer and this property makes poultry sperm to have more mid-piece bendings than other species. The in vitro assessment morphological sperm defects of cockerel semen include the following (Alkan *et al*., 2001):

1. Neck bending (mid piece bending).
2. Mid piece damage.
3. Acrosome damage.
    - Bending.
    - Swelling.
    - Knotting or rounding
4. Total head swelling.
5. Tail defects

2.13.2 Computer assisted semen analysis (CASA)

The CASA programme was first commercially introduced as CellSoft in 1985, the aim being an objective and automated semen evaluation technique, relative to sperm motion characteristics, morphology and sperm concentration. Scientists/technicians also use the analysis for many other purposes e.g. to test the effect of environment, toxicology; cooling and cryopreservation, DNA labeling, semen extender component analysis, gene transfer and sperm function and factors affecting spermatogenesis and epididymal function (Didion, 2008). The CASA system is generally used in reference laboratories with the main objective of assessing sperm motility and it uses image analysis to determine sperm concentration, within counting chambers. The accuracy of this system depends on the type of counting chamber used. It allows an objective assessment of different cell characteristics such as sperm motility, velocity, and morphology (Verstegen *et al.,* 2002). The CASA system is able to objectively determine the morphological parameters or distinguish sub-populations of

sperm head abnormalities, which are not measureable or visuable manually. Some parameters are measured in terms of the digital images obtained, while other parameters are calculated from the measurements recorded, e.g. straightness of movement and the percentage of motile cells (Vyt, 2007). CASA has the major advantage that this method is objective, independent of the interpretation of the technician and gives detailed information on sperm movement (e.g. progressive movement and hyperactivity). The disadvantages of CASA include that it is an expensive method, compared to the alternative of visual motility determinations. There are certain constraints when using CASA in practice, namely the essential need of validation, quality control, and standardization of the measures analyzed. In veterinary science, this method is not yet fully standardized and validated for all animal species (Verstegen *et al.,* 2002).

Semen samples have to be diluted before measurement by CASA, as trajectories cannot be measured if the sperm concentration is too high. The optimal temperature recommended for sperm motion analysis is body temperature (37-38°C), as the CASA analysis is influenced by the temperature of the measured semen. System settings are also different for sperm of different species (Vyt, 2007). CASA provides an efficient, precise, and reliable system to evaluate fertility objectively, improve artificial reproduction technologies, and develop physiological studies (Verstegen *et al.,* 2002).

2.14 Factors affecting cockerel semen quality/characteristics post ejaculation

2.14.1 Ambient Temperature

A decrease in ambient temperature after collection of semen decreases the activity (motility) of the sperm and semen should also not be exposed to the sun (Anderson, 2001). The sperm membrane is susceptible to changes in temperature, and this may affect the movement of the

sperm, causing deterioration in quality and fertilizing capacity. Therefore care must be taken to maintain the required temperatures (Senger, 2003). The ambient temperature is never constant and higher temperatures can increase the metabolism of the sperm cell, while cooler temperatures are less of a problem as it reduces the metabolic rate and slows down the sperm movement (Hafez & Hafez, 2000).

2.14.2 Semen osmotic pressure in poultry

Latif *et al.* (2005) concluded that an increase in the osmotic pressure can be ascribed to the contamination of broiler semen with urine and bacteria, which in turn results in the clumping of sperm. A 375 mOsm osmotic pressure is optimum for the short term storage of semen. However the recommended osmolarity of the Blom stain technique is lower and was quantified as 220mOsmol/kg dissolved in diluents, with composition similar to that of seminal plasma. However these hypo-osmotic conditions resulted in the swelling of the sperm head (Lukaszewicz *et al.,* 2008). The semen diluents must be isotonic, as the osmotic pressure created by the solution may be detrimental to the sperm cell (Senger, 2003).

2.14.3 Cockerel semen pH

The accessory fluids are of blood origin and contain aldose (presumably blood glucose). Cockerel semen lacks aldose and has a lower pH than the seminal fluid. If a large amount of seminal fluid is ejaculated, it will increase the semen volume, pH and glucose content and cause a decrease in the density of the semen. The pH of semen is slightly (pH 6.8) below neutral (pH 7) immediately after ejaculation in most poultry species. The loss in CO_2 may result in an increase of the pH, which may again be neutralized by the production of lactic acid during sperm metabolism (Cole & Cupps, 1977). The semen extenders used must provide the necessary buffers to prevent changes in pH, produced by the sperm metabolism.

The most common buffers used in semen diluents are Tris, sodium citrate and sodium phosphate. A change in semen pH generally affects the sperm motility negatively and an increase in pH has been associated with poor buffering capacity. The pH should be measured in the fresh sample as semen pH will deteriorate within a short period of time. The pH is best measured using litmus paper, as the pH meter can be expensive and the proteinaceous nature of semen frequently blocks the pH meter (Maule, 1962; Culty *et al*., 2002; Senger, 2003,).

2.14.4 Concentration of sperm per ejaculate

The sperm cell contains potassium (K^{+}) as a major cation, whereas sodium (Na^{++}) is the principal cation in the seminal plasma. Potassium is a natural metabolic inhibitor and by increasing the cellular concentration, it increases the ratio of potassium to sodium which again reduces the metabolic activity in the sperm. The addition of fructose will not greatly change the metabolic rate, but will extend the life span of the sperm. Excessive dilutions suppress sperm motility and the metabolic rate of the sperm (Nishiyama, 1961; Bearden *et al.,* 2004).

2.14.5 Gonadotrophic hormones

Bearden *et al*. (2004) reported testosterone and other androgens to suppress the sperm metabolic rate, but the concentration of the androgens found in a male system apparently has no permanent effect on sperm viability. There are fluids produced in the female tract which also increase the metabolic activity and hence motility of the sperm and it is thought that estrogen is involved in the development of the female tract, but other unidentified factors may also be involved. The increased metabolic activity of the sperm cell in the female tract is likely to increase sperm motility, which increases the frequency of collusions between the sperm cell and the oocyte in the oviduct.

2.14.6 Gasses

A low concentration of CO_2 stimulates aerobic metabolism of the sperm. The metabolic rate is suppressed if the partial pressure exceeds 5%. O_2 is necessary for aerobic metabolism, but higher levels of O_2 may be toxic, and depress the metabolic rate. This factor is not likely to occur in the laboratory, unless the O_2 or CO_2 is bubbled through the semen. Anaerobic sperm metabolism can occur under nitrogen, hydrogen, or helium gasses with no effect on the metabolic rate (Bearden *et al.,* 2004).

2.14.7 Photoperiod

According to Bearden *et al.* (2004), the lighting in the laboratory can suppress the metabolic rate, motility, and fertilizing capacity of the sperm. A greater effect was observed when semen was in contact with O_2. The enzyme catalase will prevent the harmful effect of light or photoperiod. This demonstrates that light causes a photo-chemical reaction in the semen that result in the production of hydrogen peroxide, which is detrimental to the sperm. Semen should be protected from light and also against direct sunlight.

2.14.8 Antimicrobial agents

There are a variety of bacteria that could contaminate semen after collection and this can be minimized by cleaning the area around the sex organs and using sterile equipment. Gentamicin, Tylosin and Linco-Spectin are generally added to the semen during processing and storage to limit bacterial growth. These agents have not demonstrated any direct effect on metabolic rate of the sperm. Some organisms are not pathogens, but do compete with semen for nutrients and produce metabolic by-products that may have an adverse effect on the viability of the sperm. The antibacterial agents may extend the fertile life of the semen by

controlling bacterial growth, thus saving energy substrates for sperm metabolism and maintenance (Etches, 1996; Bearden *et al.,* 2004).

2.15 Preservation of sperm cells

2.15.1 Short Term Cockerel Semen Preservation

Semen diluents are currently being used for both short and long term storage of domestic fowl semen. These extenders are being commercialized to improve the general reproductive effectiveness of the cockerels and lower the cost of AI. The development of semen diluents initially began with the use of NaCl (saline) solutions. Now complex diluents containing different osmotic regulators, energy sources and buffers are being used (Bootwalla, & Miles, 1992). The most common practice for short term fowl semen storage (hours to days at a temperature of -4˚C) requires the suspension of sperm in a suitable extender to maintain the sperm viability, in vitro. Assessment of diluted and undiluted stored cockerel semen revealed that the application of extenders is essential to sustain sperm quality. It was established that diluted fowl semen could be stored for up to 24h, without impairing the viability and fertilizing capacity of the sperm (Siudzinska & Lukaszewicz, 2008b).

Several other factors play a role in sustaining the quality of semen during storage over time e.g. the diluents used in semen extension and storage conditions e.g. time, aeration and storage temperatures. It is known that sperm motility and the fertilizing capacity of undiluted raw fowl semen stored in vitro usually decreases within 1h after collection (Dumpala *et al.,* 2006). Therefore, to store cockerel semen, the type of diluent and storage temperature is very crucial. Generally an extender will facilitate semen handling procedures, particularly during collection and evaluation, by maintaining the sperm viability, but preventing their activation. For semen maintained at 41°C and diluted with Beltsville Poultry Semen Extender (BPSE) or

Minimum Essential Medium (MEM) there were quadratic and linear increases in the percentage dead sperm over time, while a drastic linear increase existed for undiluted semen. There was thus a linear decrease in sperm quality index (SQI) for undiluted and semen diluted in MEM over time (4°C). However, for semen diluted with only BPSE, there was a linear increase (Dumpala *et al.,* 2006). Extenders serve to also protect the sperm cells from chemical and physical changes and contamination in their environment and provide more favourable conditions for fertilization (Chulhong & Chapman, 2005).

2.15.2 Long Term Cockerel Semen Preservation

Blesbois (2007) defined cryopreservation as a non-physiological method involving higher levels of adaptation of biological cells to osmotic and thermal shock, occurring during the cooling/freezing and thawing processes in the sperm cell. The possibility of dilution and storage of avian sperm could facilitate the objectives of poultry breeders, enabling them to ship semen to remote production units and to inseminate large groups of females and thus increase the use of superior males (Siudzinska & Lukaszewicz, 2008b). However, associated protocols remain troublesome, due to the need for many steps in the freeze-thawing process (Blanco *et al.,* 2000). The sperm cells of birds contain very little cytoplasm and posses large plasma membranes and also contain a variable number of mitochondria and a nucleus containing the chromatin. The use of glycerol as a cryoprotectant in the freezing process pioneered the success of cryobiology and resulted in high cryopreservation success rates being obtained within a wide range of species. Significant differences exist between domestic bird species, such as the turkey, broiler and layer type of chickens, in terms of sperm viability and functionality after cryopreservation. The fertility rate of chicken frozen/thawed semen is e.g. consistently higher than that of cryopreserved turkey semen (Long, 2006).

Cell damage occurring during the freeze-thawing procedures mainly affect the plasma and mitochondria and in the worst case scenario the nucleus of the sperm. The duration of the fertility is also affected and is a very important factor in the bird's reproduction, due to the long term in vivo storage of the semen. Cell structure and metabolism of the sperm during cryopreservation is affected mainly by the interaction of the sperm and the internal cryoprotectant agent, added to limit temperature stress, the temperature curve of freezing and the thawing and packaging device (Blesbois, 2007).

Cockerel semen cryopreservation is a technique that is currently still very much in the experimental phase and there are many problems being encountered that could be associated with this technique. Aspects could be the failing to select the most suitable diluent for low temperature storage, how to identify cryoprotectants that are able to limit sperm cell damage caused by freezing, the selection of optimal freezing temperatures and how to achieve an acceptable cooling rate and also methods of separating the cryoprotectant from the seminal material and the degree of dilution of the seminal material before fertilization. Finally there is also the aspect of how to choose the most suitable storage devices during refrigeration and freezing.

The cryopreservation of chicken semen has generally been successful in terms of the fertility rate obtained after insemination. Where hens are inseminated at 3-day intervals or for 3 consecutive days, a higher fertility rate of approximately 90% has been recorded. This semen was cooled at a rate of 1°C /min to -35°C, followed by the immersion into liquid nitrogen. Similar success rate have been achieved when dimethyl acetamide (DMA) was used as cryoprotectant and artificial insemination was performed after pellet freezing (Blanco *et al*., 2000).

2.15.3 Poultry Semen Extenders

Diluents are generally buffered salt solutions used to extend semen, maintain the viability of the sperm in vitro, and maximize the number of hens that can be inseminated by increasing the volume. Poultry semen is generally viscose and highly concentrated, and contains billions of sperm/ml, and thus needs to be diluted for AI purpose (Donoghue & Wishart, 2000). Both hypertonic and hypotonic extenders reduce the metabolic activity of the sperm, and could disrupt the cell membrane integrity that leads to the clumping of the sperm (Latif *et al.,* 2005).

Glutamic acid is the most prominent anionic constituent in avian seminal plasma, and is a standard component of all semen diluents. Basic characteristics common to all diluents include the maintenance of pH, osmolarity and the provider of energy for the sperm. The motility and metabolic rate of sperm can thus be altered by decreasing the diluent below pH 6.0. So for example a low pH reduces the sperm motility and a high pH increases the metabolic rate in vitro (Donoghue & Wishart, 2000). Modified Ringer's solution with the following composition of sodium chloride (68g), potassium chloride (17.33g), calcium chloride (6.42g), magnesium sulphate (2.50g), sodium bicarbonate (24.50g) and distilled water, can be used to dilute poultry semen (Martin, 2004).

The extenders or diluents used in poultry production must provide buffers to prevent changes or stabilize the semen pH. The most common buffers used are Tris, sodium citrate and sodium phosphate. The sperm membrane is, as already mentioned, also susceptible to changes in temperature, and this may affect the movement of the sperm, causing deterioration in semen quality. Care must therefore be taken to maintain the diluted semen at the prescribed temperatures. The diluents must be isotonic as osmotic pressure created in the solution could be detrimental to the sperm e.g. dehydration of the cell (Senger, 2003). Diluents or extenders

must also protect the sperm against cold shock injury during freezing, provide the necessary nutrients for sperm metabolism, and contain penicillin and streptomycin to control microbial contaminants. Further diluents must contain a cryoprotectant to protect the sperm from injury during freezing and thawing, and must preserve the viability of the sperm, with a minimum drop in overall fertility (Bearden *et al*., 2004). There are breed differences regarding the storability of cockerel semen. Thus semen collected from roosters of different breeds require different storage conditions to retain their viability and a need therefore exists to adjust extenders for individual breeds (Siudzinska & Lukaszewicz, 2008a).

2.15.4 Beltsville Poultry Semen Extender (BPSE)

BPSE was developed by Sexton (1977), (see Table 2.4) being one of the first people to discover the importance of the dilution of the insemination dose required for optimum fertility following short term semen storage in poultry. The Beltsville cryopreservation technique entails three phases, namely, a pre-freeze, freeze and thawing phase. The importance of the pre-freeze stage lies in the diluent, cryoprotectant agent and the holding temperature. It was formulated to be used in frozen cockerel semen and it is physiologically balanced to support sperm viability, in-vitro. The BPSE is strongly buffered, to reduce the cytotoxic effects of the cryoprotectant. BPSE is generally used with DMSO, TES and potassium phosphate being components of the BPSE extender of poultry semen, which are important in reducing the cytotoxic effect of DMSO and the natural salts (magnesium chloride, potassium citrate, and sodium acetate). For freezing it is important to know the final temperature and cooling rate, as these affect the survival of the sperm. In the Beltsville method, cockerel semen is slowly cooled from +5 ˚C to -20˚C at 1˚C/min. A cockerel semen temperature of -80˚C can be obtained by the cooling rate of 50˚C/min, while thawing has been achieved by placing semen straws in an ice bath (+2˚C). When inseminating White

Leghorn hens, weekly with $20x10^6$ sperm diluted in BPSE, a fertility rate of 88% was recorded. The main objective of this specific extender, being to establish if this diluent would yield a better recovery of viable sperm following thawing, (Sexton, 1979; Bootwalla & Miles, 1992).

Table 2.4 The composition of the Beltsville Poultry Semen Extender (Sexton, 1977)

Component	Level g/l	Primary function
Dipotassium phosphate	12.70	Buffer
Sodium glutamite	8.61	Chelator
Fructose	5.00	Metabolic substrate
Sodium acetate	4.30	Osmotic balance
Tes*	1.95	Buffer
Potassium citrate	0.64	Osmotic balance
Monopotassium phosphate	0.65	Buffer
Magnesium chloride	0.34	Osmotic balance

*N-Tris (Hydroxymethyl) Methyl-2-Aminoethane sulfonic acid

2.16 Poultry Semen Cryoprotectants

2.16.1 Cryoprotectants

Cryoprotectants are agents that protect the sperm against temperature fluctuations. These agents are very important additives and generally include cryoprotectants such as glycerol and dimethyl sulfoxide (DMSO). Cryoprotectants currently used for cockerel semen freezing include glycerol, dimethylphoxide, dimethylacetamide, ethyleneglycol, dimethylformamide and propyleneglycol. Glycerol, DMSO, DMA and DMF, have been added to give final

concentration (4, 6, 8 or 10% v/v) after the first equilibration. The 10% DMA and DMSO were found to be the best cryoprotectants for cryopreservation of cockerel semen (61.25-73.12% motility), with 8% DMF and glycerol being significantly higher than that obtained with DMF (58-68% motility) and glycerol levels of 4, 6 or 10% (Tselutin *et al.,* 1999; Donoghue & Wishart, 2000; Han *et al.,* 2005).

2.16.2 Dimethyl sulfoxide (DMSO)

One of the most crucial steps in the successful cryopreservation of poultry semen is the choice of the cryoprotectant and its function during the process. A variety of cryoprotectants, e.g. glycerol, DMSO, and dimethyl acetamide (DMA) have been used in the past. Most given cryoprotectants have been used with reasonable satisfactory fertility results, provided that the hens are inseminated with sufficient numbers of thawed sperm (Tselutin *et al.,* 1999). DMSO has been used as an alternative improved cryoprotectant, to glycerol (Blanco *et al.,* 2000). DMSO has a buffering capacity and enters the sperm cell fast and is very effective when slow freezing semen (Bearden *et al*., 2004). DMSO is also beneficial, as it serves as a dehydrating agent (Hafez & Hafez, 2000). It is composed of the buffers Tes and potassium phosphate to minimize its cytotoxic properties, and the neutral salts magnesium chloride, potassium citrate and sodium acetate. When DMSO is added to an extender, the viability of the sperm is maintained if the diluted semen is gradually cooled to +5°C before freezing (Sexton, 1979). Results show that a diluent containing 10% DMSO to be more effective than others (Han *et al.,* 2005).

2.16.3 Glycerol

Glycerol is the most common cryoprotectant used for cryopreservation of sperm. It is generally used in bovine semen cryopreservation and is utilized, because of its quality in

binding water and decreasing the freezing point of the solution (Senger, 2003). Glycerol penetrates the sperm cell and dehydrates the cell partially, thereby reducing the risk of water crystallization (Bearden *et al*., 2004). The percentage of glycerol added to the diluent is very important for the survival of the sperm cell and its motility. The cockerel sperm metabolize glycerol quickly and have the ability to become reversibly immobilized under a variety of conditions during cryopreservation such as lack of oxygen and reducing the sugar level in the diluent (Cole & Cupps, 1977). Glycerol in poultry has been shown to have a contraceptive effect, therefore, semen has to be washed free of glycerol after thawing prior to AI (Bellagamba, *et al*., 1993).

The percentage of glycerol added to diluted semen depends on the type of diluent used. 7% glycerol was found to be optimal when added to a solution containing yolk citrate, whole milk and yolk tris diluters after final dilution. Skim milk as diluent performs well when 10% glycerol is added (Bearden *et al*., 2004). Egg yolk may affect the effectiveness of glycerol and therefore the amount of egg yolk added generally determines the percentage of glycerol to be added. The higher the percentage of egg yolk in a diluent, the higher the amount of glycerol to be added (Maule, 1962).

The mechanism by which glycerol exerts a contraceptive action has not been clarified. It interferes with fertilization, so its action is exerted after AI and its contraceptive effect is exerted on the vaginal cells. Hence dialysis or centrifuging and redilution of the semen sample are necessary before AI. The addition of glycerol (15%) causes instability in the cell membrane, with a consequent change in its permeability (Bellagamba, *et al*., 1993).

2.16.4 Other semen cryoprotectants used in cockerel semen cryopreservation

There are other cryoprotectants used to preserve cockerel semen e.g. dimethyl formamide (DMF) and DMA. The effects of these different concentrations of cryoprotectants including dimethyl sulphoxide, dimethyl acetamide, and dimethyl formamide in the cryopreservation of poultry semen, have been evaluated (Han *et al.,* 2005). According to Tselutin *et al.* (1999), when semen is frozen in pellets, at very high cooling rates, DMA performed better than glycerol as a cryoprotectant in cockerel semen as evidenced by the fertility rates obtained. In contrast, when straws and low freezing rates were utilised, glycerol gave better fertility results (57.4-67.4%). However, these results were not as high as those obtained when using DMA as a cryoprotectant and freezing in pellets (73.7-94%).

2.17 Conclusions

The application of gamete cryopreservation in poultry has been practiced for decades. However for many decades scientists failed to record genetic gain and this resulted in a loss of valuable alleles the reason being poor fertility following the use of frozen semen. More recently technology development, has resulted in better fertility rates, but this technique has been underutilized in poultry because of a lack of interest by poultry breeders as such (Gill & Barbato, 2001, Blesbois, 2007). Cockerel semen evaluation is an essential aspect in the assessment of the breeding soundness of any male and the relationship between semen volume, sperm motility, sperm concentration, pH and colour are very important in semen evaluation. These parameters, to a large extent, determine the fertility potential of semen (Peters *et al.,* 2008). It is generally accepted that the evaluation of the quality of semen helps to predict the suitability of sperm to withstand the processes of freezing and thawing. In general cryopreservation of semen damages the sperm cells and systematically decreases the quality of the sperm (Blesbois *et al.,* 2008). The difficulty in identifying ideal cryoprotectants

which will maintain a good fertilizing capacity of sperm after the freeze/thaw process poses a hurdle in the success and development of the technique of cryopreservation of chicken semen (Bellagamba *et al.,* 1993). Moreover, the cryopreserved semen can be transported over long distances between farms, throughout the year and contribute to accelerate genetic progress (Lukaszewicz, 2002).

Chapter 3

General Material and Methods

3.1 Study area and period

The study was conducted at the Glen Agricultural Development Institute (ARC) and at the University of the Free State, Bloemfontein campus, South Africa. Situated at a latitude of 29.10° South, longitude of 26.29° East and an altitude of 1351m above sea level. The study was undertaken between May and September, 2009 (end of autumn to onset of spring).

3.2 Experimental chickens

Cockerels of 4 different breeds (layers plus dual purpose chicken genotypes) of the domestic fowl, namely the Rhode Island Red (RR), Potchefstroom Koekoek (PK), New Hampshire (NH) and White Leghorn (WL) were used as the semen donors in the study (Plate to 3.1, 3.2, 3.3, and 3.4). Each breed was represented by 7 sexually active mature cockerels (9 months of age) and 80 mature hens (8 months of age) of the four different breeds (White Leghorn, Potchefstroom Koekoek, Rhode Island Red and New Hampshire), with 20 hens per breed being used in the study. Both cockerels and hens were maintained in cages for the entire period of the study.

3.3 Housing and feeding of the cockerels and hens

The birds (male and female) were managed intensively in a battery cage system and each bird was kept in an individual cage (60 x 50 x 75cm). The cockerels were allowed a week of adaptation prior to the onset of semen collection, and were trained to respond to the massage technique prior to semen collection (Burrows and Quinn, 1937). The hens were given an adaptation period of 2 weeks. All birds were provided with laying mash diet containing 180g

crude protein, 11.3MJ ME and 30g calcium per kg diet, with water available *ad libitum* throughout the observation period.

Plate 3.1 Rhode Island Red cockerels used for semen collection

Plate 3.2 Potchefstroom Koekoek cockerels used for semen collection

Plate 3.3 New Hampshire cockerels used for semen collection

Plate 3.4 White leghorn cockerels used for semen collection

Plate 3.5 White Leghorn and Potchefstroom Koekoek hens used for AI with fresh and frozen semen

Plate 3.6 Rhode Island Red and New Hampshire hens used for AI with fresh and frozen semen

3.4 Semen collection and evaluation

The four different chicken breeds, namely the Rhode Island Red (body weight of 2.5 to 3kg), Potchefstroom Koekoek (body weight of 2.7 to 3.1kg), New Hampshire (body weight of 2.7 to 3.1kg) and White Leghorn (body weight of 1.9 to 2.2kg), were used for semen collection and evaluated in this trial. Semen was collected from each cockerel twice a week by the abdominal massage method (Burrows and Quinn, 1937). These semen collections were performed on Mondays and Thursdays between 9:00 and 11:00, from May to June (end of spring to onset of winter) for a total period of 5 weeks in the first trial of the experiment. During semen collection, the semen collection tubes were maintained at 42˚C, until the semen was microscopically evaluated for semen quality.

The following parameters were evaluated to characterize each cockerel's semen quality: Firstly the ejaculate volume and colour of the sample was macroscopically evaluated, immediately after collection, and recorded directly from the semen collection tube. The pH of the fresh semen sample of each cockerel was measured with the aid of litmus paper (Merck KGaA, 64271 Darmstadt, Germany), while sperm mass motility, scored on a scale of 0 and 5, was evaluated subjectively under a light microscope (X40 magnification), giving a general indication of the type and intensity of the sperm movement and the impact of movement on the number and size of the sperm agglutinates (Blesbois *et al*., 2008). Semen was then diluted 1:100 (semen: extender) using the modified Beltsville Poultry Semen Extender (mBPSE) and the sperm motility estimated using a phase-contrast microscope (X40 magnification), a subjective method of evaluating individual sperm movement by counting a total of 100 sperm, being classified as motile or immotile and expressing it as a percentage (%).

The sperm concentration of an ejaculate was determined by using a Neubauer hemacytometer and the sperm count performed as described by Hafez and Hafez (2000). Briefly, a volume of

10µl semen was diluted with 990µl Sabax water, in an Eppendorf flask and stored in a refrigerator, before counting, to immobilize the sperm. To determine the percentage live sperm, an eosin/nigrosin stain was used for the microscopic morphologic observations (Table 3.1). Here a 10µl drop of fresh semen was mixed with 200µl of eosin-nigrosin stain, and a smear made of the mixture on a slide and examined under X1000 magnification. Approximately 100 sperm were counted to determine the percentage dead-live sperm (Lukaszewicz *et al*., 2008). There are currently a variety of protocols available for semen stain preparations and methods of making semen smears. The eosin-nigrosin stain was selected due to the more acceptable results (Plate 3.7) being obtained using this technique when e.g. compared to the other staining methods such as Blom and Morisson (Lukaszewicz *et al*., 2008).

Table 3.1 The chemical composition of the eosin-nigrosin stain used for semen evaluation (Lukaszewicz *et al.,* 2008)

Chemicals	Amount (g)
Sodium glutamate	1.7351
Potassium citrate	1.280
Sodium acetate	0.851
Magnesium chloride	0.686
Eosin	1.0008
Nigrosin	5.0002

***All components were dissolved in SABAX water**

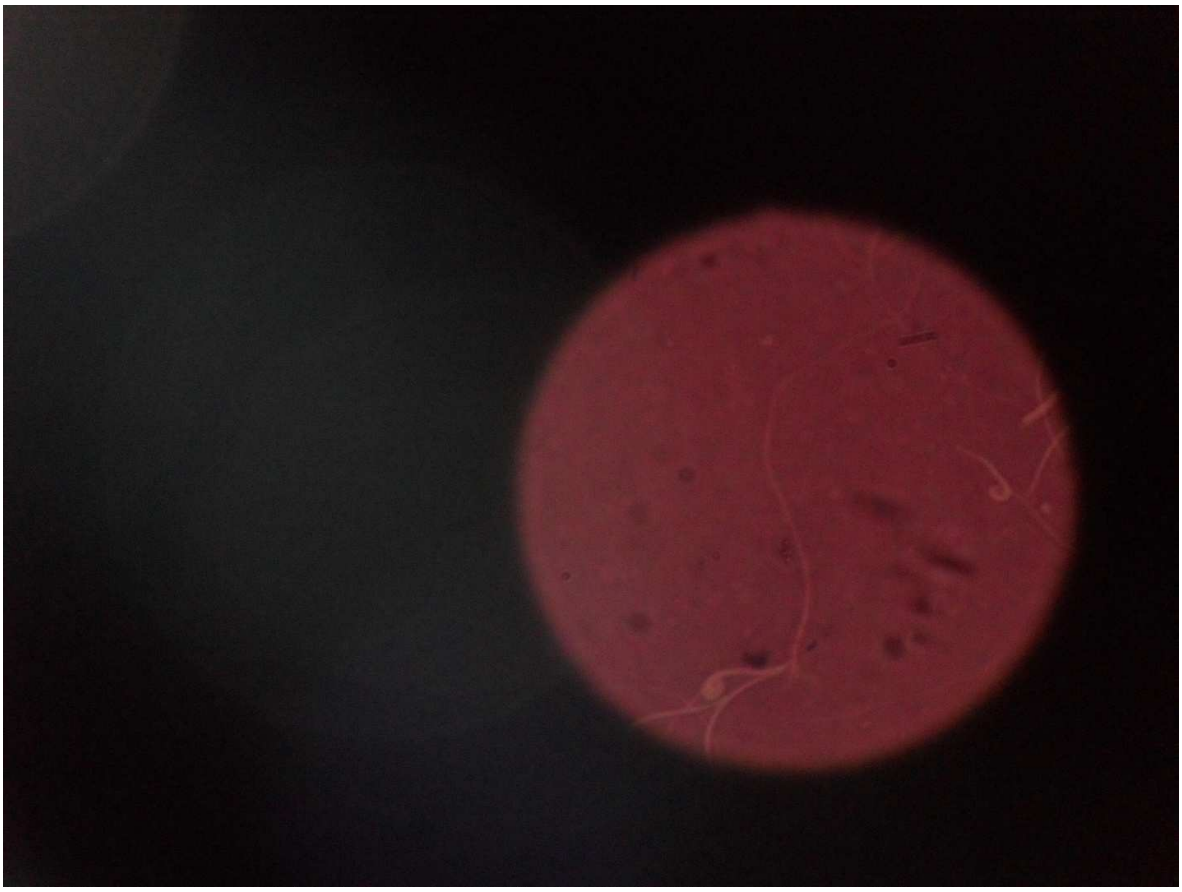


Plate 3.7 Slide of semen stained with eosin nigrosin

3.5 Cockerel Semen Cryopreservation

Cryopreservation of semen is a non-physiological technique, entailing the need for higher levels of adaptation of the biological cells to osmotic and thermal shock on the sperm cell membrane and content, such as e.g. those occurring during the cooling/freezing and the thawing procedures of the sperm cell. Glycerol is generally known as an effective cryoprotectant used for fowl semen, but it has been found to have a contraceptive effect following intravaginal insemination in the chicken or turkey (Woelders *et al*., 2006). Subsequently dimethyl acetamide (DMA) or dimethyl sulfoxide (DMSO) has been used as a cryoprotectant to replace glycerol during cryopreservation, with the aim of overcoming this contraceptive action of glycerol as it appears to interfere with fertilizing process Its action is

exerted after the sperm are deposited in the female genital tract, although these agents again also have certain cytotoxic effects on the sperm cell.

Semen was collected from 21 cockerels (7 cockerels died) (4 breeds) three times per week for a period of 3 weeks in the second trial of the experiment. All semen was collected at room temperature in graduated semen collection tubes and maintained at 42°C in a warm bath, until microscopically evaluated for quality. Contaminated semen samples (containing blood or fecal material) were discarded, while all ejaculate volumes were recorded directly from the graduated semen collection tubes.

The individual cockerel ejaculates were diluted using the modified BPSE diluent before cryopreservation, as previously described by Sexton (1977), and modified by the addition of glucose instead of fructose (as glucose was more readily available). Firstly the semen was slowly diluted with fraction A of the modified BPSE diluent (Table 3.2), in the ratio of 1:1 (v/v), evaluated and maintained at room temperature in the semen collection tubes for 20 minutes, and then equilibrated for 1h at 4°C. The semen was again diluted in the ratio of 1:1 (v/v) with fraction B of a modified BPSE + 10% DMSO diluent and equilibrated for a further 1h at 4°C (Han *et al*., 2005). Before the actual semen cryopreservation, cooled semen samples were frozen as pellets on a block of dry ice in indentations on the surface of the dry ice (Plate 3.8). Cooled diluted semen aliquots of 0.2 ml were transferred into the indentations on the dry ice, using a 1 ml syringe. The semen suspension formed a pellet within 2 to 3 minutes. The resulting frozen semen pellets were then plunged into liquid nitrogen (-196°C), for long term storage (Plate 3.9).

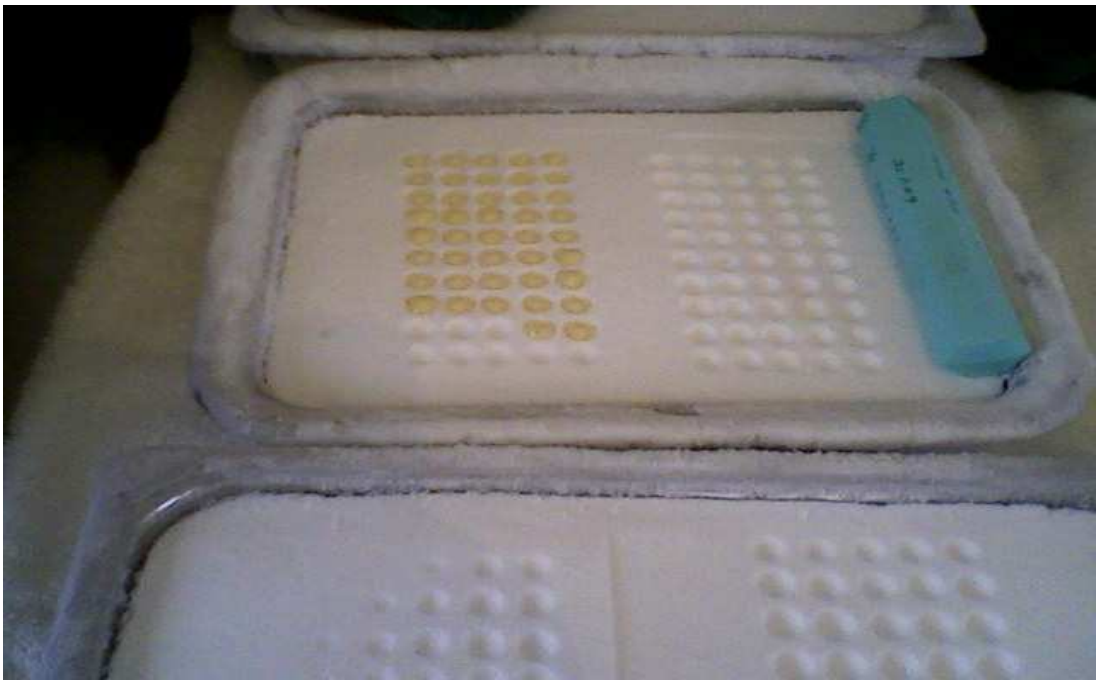


Plate 3.8 Semen pellets on dry ice

Plate 3.9 Pellets plunged in Liquid Nitrogen before storage in Liquid Nitrogen tank

For thawing the frozen cockerel semen pellet was placed into a test tube in a water bath at 60˚C (Plate 3.10), and the tube shaken continuously, until complete thawing of the pellet. The sperm motility was microscopically evaluated (X40 magnification), immediately after

thawing and the sperm viability (percentage live sperm) was determined, using an eosin-nigrosin stain (see 3.4). A total of 100 sperm cells were counted per microscope slide, and the results expressed as the percentage of live sperm and morphologically normal sperm cells. All dead sperm cells absorbed the red stain and consequently coloured light red, while the live sperm remained transparent.

Table 3.2 The composition of the modified Beltsville Poultry Semen Extender used in cockerel semen cryopreservation (Sexton, 1977).

Component	Level g/l	Primary function
Dipotassium phosphate	1.27	Buffer
Sodium glutamite	0.867	Chelator
Glucose	0.5	Metabolic substrate
Sodium acetate	0.43	Osmotic balance
Tes*	0.195	Buffer
Potassium citrate	0.064	Osmotic balance
Monopotassium phosphate	0.065	Buffer
Magnesium chloride	0.034	Osmotic balance

*N-Tris (Hydroxymethyl) Methyl-2-Aminoethane sulfonic acid

Plate 3.10 Water bath used for thawing the frozen cockerel semen pellets

3.6 Artificial Insemination (AI) in the chicken

AI of all hens was performed between 12:00 and 15:00, as most hens have an egg in the oviduct in the morning, thus obstructing the free passage of semen to the ovary. AI was performed using 1.0 ml syringes (Plate 3.11) for the deposition of the semen as described by Sadanand *et al*. (2004). During the insemination process the cloaca was turned inside out and 0.2 ml of the semen was deposited inside the oviduct. AI was performed for a period of two weeks. Both fresh and frozen semen were microscopically evaluated for sperm motility prior to insemination.

For fresh semen AI, hens were inseminated once in a week (Tuesday). Immediately after collection and evaluation, the semen from each of the Rhode Island Red, Potchefstroom Koekoek, New Hampshire and White Leghorn cockerels was inseminated into 10 of the Rhode Island Red, Potchefstroom Koekoek, New Hampshire and White Leghorn hens,

respectively. For frozen semen AI, hens were inseminated twice in a week (Tuesday and Thursday), with pellets for Rhode Island Red, Potchefstroom Koekoek, New Hampshire and White Leghorn cockerels being thawed at the time of insemination, and immediately inseminated to the 10 hens of Rhode Island Red, Potchefstroom Koekoek, New Hampshire and White Leghorn breeds, respectively.

Plate 3.11 AI performed using a 1.0ml syringe

3.7 Collection and incubation of the eggs

The day of artificial insemination (AI) was recorded as day 0 of fertilization in the hen. After a single insemination with both fresh and frozen semen, eggs were collected daily from the 2nd day post insemination, for a period of 7 days after AI. This was taken as the first day of the production of fertile eggs. The eggs were labelled in order to identify the different breeds (Rhode Island Red, Potchefstroom Koekoek, New Hampshire and White Leghorn) and the day of collection. All eggs were collected in the afternoons (between 13:00 and 15:00) and

stored at room temperature (20 - 25˚C), prior to incubation for 18 days, at 37˚C. Cracked and dirty eggs were not incubated and were discarded.

On the 18th day of incubation all eggs (n = 973) were candled, to identify the fertile eggs. (Islam *et al.,* 2002). Only fertile eggs were then transferred to the hatchery for a period of 3 days. The eggs that did not hatch were cracked open to determine the possible cause of infertility or non-hatching. The fertility, number of hatched and dead chicks were counted (Plate 3.12) and recorded for all the 4 breeds including treatments (fresh and frozen AI). The following temperatures and humidity were maintained during incubation period:

a) Setting temperature (37˚C) of eggs up to 18 days of incubation.

b) Hatching temperature (37˚C) of chicks from 18-21 days of incubation.

c) Setting Relative humidity: 60% up to 18th day of incubation (Banks, 1979).

d) Hatching Relative humidity: 60% from 18-21 days of incubation (Banks, 1979).

Plate 3.12 Hatched chicks from fresh and frozen semen

3.8 Statistical Analyses

Statistical analyses of the sperm characteristics of fresh and frozen semen was performed using the SAS ANOVA program and Tukey's Studentized Range (HSD) for repeated measures. The F values were considered significant at a confidence level of $P<0.05$. The Tukey test was performed to test for significant differences between treatments (SAS, 2006).

Chapter 4

The viability of semen from different cockerel breeds generally used in South Africa

4.1 Introduction

The main function of the cockerel in poultry production is the production of male gametes, (sperm cells) during copulation, to deposit the semen into the hen's cloaca. On average a cockerel produces approximately 3 x 10^6 sperm daily in an ejaculate of approximately 0.7ml semen. When compared to other poultry birds, this quantity is dependent of the breed, age of the male, the individual itself, season, light and certain environmental and managerial factors (Tuncer *et al.,* 2008). There are generally differences in the volume and concentration of the ejaculate between breeds, being mainly due to the genetic make-up of the individual breed. In addition, variations in semen production can also be attributed to seasonal differences in production management practices, semen collection skills and the time of semen collection (Peters *et al.,* 2008).

Reproductive efficiency in broiler/layer chickens may be affected by a variety of interacting variables. These include the combined effect of sexual behaviour and social status, feeding regimes and the impact of selection for growth and egg production. Reduced male fecundity in the poultry industry may be attributed to amongst others, the selection for the genetic improvement of growth rate (Froman, 2006). However, currently artificial insemination (AI) can be implemented on chicken farms, once natural mating is no further possible in birds so e.g. birds with superior feed conversion qualities usually develop a too large body size and other age-related constraints (Alkan *et al.,* 2001).

Semen quality remains one of the most important characteristics that determine fertility in the male. It is important to characterize the quality parameters in terms of semen volume, semen colour, sperm concentration, sperm motility, sperm viability and morphology. In all poultry species, semen quality parameters vary with the age of the male, leading to a progressive decline in fertility with age (Kotlowska *et al*., 2005). Currently many methods are used for assessing semen quality, e.g. sperm motility assays and sperm quality indexes, while estimating the fertilizing capacity of sperm is also a possibility. Some methods however, are highly subjective, while others require special sophisticated laboratory apparatus (Lukaszewicz *et al.,* 2008). This study focuses on the comparative evaluation of semen quality (using standard techniques) in different breeds of cockerels commonly farmed in Southern Africa**,** with the ultimate aim of improving the production efficiency (meat and egg production).

4.2 Material and Methods

Four different poultry breeds namely; the Rhode Island Red (body weight between 2.5 to 3kg), the Potchefstroom Koekoek (body weight between 2.7 to 3.1kg), New Hampshire (body weight between 2.7 to 3.1kg) and the White Leghorn (body weight between 1.9 to 2.2kg), were used for the collection of semen at regular intervals overtime for microscopic sperm evaluation. All cockerels were kept in individual battery cages, provided with a diet of laying mash and water ad libitum (see 3.3). Semen was collected from each cockerel twice weekly, using the abdominal massage method (Burrows and Quinn 1937). These semen collections were performed on Mondays and Thursdays (in the morning between 9:00 and 11:00), from May to June (end spring and onset of winter) for a total period of 5 weeks. During semen collection, the semen collection tubes were maintained at 42˚C, until microscopically evaluated for semen quality. The following parameters were evaluated to characterize each

cockerel's semen quality. Ejaculate volume and colour were visually evaluated immediately after collection, directly from the graduated semen collection tube. The pH of the fresh semen sample of each cockerel was then measured with the aid of litmus paper. The sperm mass motility (scored between 0 and 5), was determined subjectively, under a light microscope (x40 magnification), as described by Blesbois *et al*. (2008). Semen was then diluted 1:100 (semen: extender) using the modified Beltsville Poultry Semen Extender and the individual sperm motility estimated using a phase-contrast microscope (x40 magnification), by counting a total of 100 sperm cells, classified as motile or immobile and expressed as a percentage (%) (See 3.4).

The sperm concentration of an ejaculate was calculated by using a Neubauer hemacytometer and the sperm count determined as described by Hafez and Hafez (2000). Briefly, a volume of 10µl semen was diluted in 990µl Sabax water in an Eppendorf flask and stored in a refrigerator (4˚C) before counting, to kill the sperm and make them immobile. To determine the percentage live sperm, an eosin-nigrosin stain was used in the microscopic morphologic observations. Here a 10µl drop of fresh semen was mixed with 200µl of an eosin-nigrosin stain. A smear was made of the mixture on a slide and the sperm examined under x1000 magnification. Here approximately 100 sperm were counted to determine the percentage dead/live sperm (see 3.4).

4.3 Results

The evaluation of semen quality is of utmost importance from the point of implementing AI and obtaining fertilization. It is known that sperm originating from different breeds of chicken vary in many respects. Semen characteristics of the 4 breeds of chicken are set out in Table 4.1, 4.2 and 4.3. The differences in the body weight for the different breeds of cockerel, ejaculate volume, sperm motility, sperm concentration, semen pH, estimated sperm motility,

percentage live sperm and dead sperm, normal and abnormal sperm (head, mid-piece, tail and other sperm abnormalities) between all collected semen samples were statistically different ($P<0.05$). The means as set out in Table 4.1, revealed no significant difference between the body weight of the Rhode Island Red, Potchefstroom Koekoek and New Hampshire, while the White Leghorn was the lightest regarding body weight, when compared to the other breeds of chickens, (with corresponding values of 2.94 ± 0.29kg, 2.89 ± 0.37kg, 2.99 ± 0.24kg and 2.08 ± 0.24kg respectively). Similarly the Rhode Island Red cockerels produced the biggest ejaculate volume, followed by the New Hampshire, White Leghorn and Potchefstroom Koekoek (0.43 ± 0.14ml, 0.35 ± 0.12ml, 0.29 ± 0.13ml and 0.25 ± 0.11ml respectively).

Table 4.1 The mean (±SD) seminal characteristics of semen collected from 4 cockerel breeds

Breeds	**Body weight (kg)**	**Ejaculate Vol. (ml)**	**Semen pH**	**Sperm Motility(0-5)**
Rhode Island Red	2.9^{a} ±0.3	0.4^{a} ±0.1	7.6^{a} ±0.4	2.8^{a} ±0.9
Potchefstroom Koekoek	2.9^{a} ±0.4	0.3^{c} ±0.1	7.6^{a} ±0.4	3.0^{a} ±0.9
New Hampshire	3.0^{a} ±0.2	0.4^{a} ±0.1	7.7^{a} ±0.3	2.8^{a} ±0.8
White Leghorn	2.1^{b} ±0.2	0.3^{bc} ±0.1	7.7^{a} ±0.0	3.1^{a} ±0.9

$^{a\text{-}c:}$ Means within each column with different superscripts differ significantly ($P<0.05$).

The results in Table 4.2 revealed no significant difference in the recorded sperm motility, percentage live and dead sperm, while the ejaculate concentration and percentage normal sperm were significantly ($P<0.05$) different between breeds.

Table 4.2 The mean (±SD) seminal characteristics of cockerel semen collected from 4 different chicken breeds

Breeds	**Estimated Sperm motility (%)**	**Ejaculate Concentration (x10^{9}sperm/ml)**	**Live sperm(%)**	**Dead sperm(%)**	**Normal sperm (%)**
Rhode Island Red	59.6^{a} ±14.5	748.5^{a} ±475.3	81.5^{a} ±26.5	18.6^{a} ±26.8	82.5^{a} ±13.4
Potchefstroom Koekoek	61.6 ^{a} ±14.1	320.4^{c} ±286.5	75.9 ^{a} ±33.3	20.0^{a} ±29.5	83.2^{a} ±19.3
New Hampshire	58.8^{a} ±12.5	499.2^{b} ±340.8	75.6^{a} ±29.1	24.4^{a} ±29.1	77.3^{b} ±17.1
White Leghorn	63.8 ^{a} ±13.6	427.2^{bc} ±309.3	80.7^{a} ±27.9	19.2^{a} ±27.9	84.8^{a} ±9.

$^{a-c:}$ Means within each column with different superscripts differ significantly (P<0.05).

The mean abnormalities and sperm defects recorded regarding the head, mid-piece, and tail of the sperm in the 4 breeds are set out in Table 4.3. The New Hampshire breed recorded a higher percentage of sperm with head, mid-piece, and tail abnormalities, when compared to the semen of the other three poultry breeds assessed.

Table 4.3 Sperm abnormalities of fresh cockerel semen collected from the 4 chicken breeds

	Sperm Abnormalities				
Breeds	**Sperm Head (%)**	**Mid-piece (%)**	**Tail (%)**	**Other Abnormalities (%)**	**Total Abnormalities (%)**
Rhode Island Red	7.1^{a} ±9.9	8.9^{bc} ±6.2	0.9^{b} ±1.8	0.6^{b} ±0.9	17.5^{b} ±13.4
Potchefstroom Koekoek	2.9^{b} ±3.3	7.9^{c} ±5.2	0.5^{b} ±0.9	1.2^{ab} ±1.7	12.5^{b} ±8.2
New Hampshire	7.7^{a} ±9.6	12.7^{a} ±6.5	1.9^{a} ±3.0	1.5^{a} ±1.9	23.8^{a} ±16.3
White Leghorn	3.0^{bc} ±2.5	11.0^{ab} ±7.0	0.4^{b} ±0.8	0.9^{ab} ±1.2	15.3^{b} ±8.5

$^{a-c:}$ Means within each column with different superscripts differ significantly (P<0.05)

4.4 Discussion

Body weight may be a good indicator of semen volume and sperm concentration (semen production) potential in certain chicken breeds. Generally, poultry breeds with heavier body weights have been found to have larger testes and produce more sperm cells during spermatogenesis and thus ultimately resulting in a higher semen concentration (Adeyemo *et al*., 2007). However, it was also observed that males with a higher body weight produce ejaculates of greater volume, but a lower sperm concentration. In this study the body weight of the cockerels showed no effect on the semen volume, as the White Leghorn with the lightest body weight (2.08 ± 0.24kg) recorded a higher ejaculate volume when compared to e.g. the Potchefstroom Koekoek, with a heavier body weight (2.89 ± 0.37kg). The overall reported average ejaculate volume of cockerel has been quoted as 0.7 ml for different poultry breeds (Tuncer *et al.,* 2008). All the breed ejaculate volumes recorded in the current trial were less than this 0.7 ml, and there may be many reasons contributing to these lower semen volumes, e.g. breed, age, individual differences, body weight, excessive stimulation, season and many environmental factors including management and the human factor. These results of ejaculate volume are similar to the results obtained by other researchers and are within the acceptable range for especially poultry AI. Other researchers obtained a mean volume of 0.28 ± 0.14 ml, which is again lower than the results obtained in this study (Bah *et al*., 2001; Galal, 2007; Peters *et al.,* 2008; Tuncer *et al.,* 2008).

The pH of the cockerel semen from the 4 breeds studied was slightly alkaline and ranged from 7.55 ± 0.39 to 7.69 ± 0.04. These results are all within the range generally reported for poultry semen. The pH of cockerel semen recorded by other researchers is 7.02 ± 0.01, 7.4 ± 0.2 and 7.68 ± 0.01. A factor that could play a role is the technique of semen collection and

stimulation of the accessory sex glands. The accessory sex gland fluid is generally alkaline (Bah *et al*., 2001; Peters *et al*., 2008; Tuncer *et al*., 2008).

The colour of the cockerel ejaculates did not differ significantly between the 4 breeds studied, being creamy-white and showing that the massage technique used may be acceptable for cockerel semen collection, in order to obtain good quality semen. These observations were consistent with Peters *et al.* (2008). It was also observed that variations in semen colour may arise in part due to the presence of contaminates or as a result of low sperm concentrations (Machebe & Ezekwe, 2005).

The differences in the semen concentration of the ejaculates may be attributed to the fact that the breeds used were from different genetic lines and differ in production traits. The general sperm concentration may further depend on the factors such as breed, age, season, individual performance and semen collecting frequency. Researchers have also found the diluent or extender to have a significant influence on the sperm motility and viability. Sperm motility was found to be lower when semen was diluted with the cryoprotectants dimethyl sulfoxide (DMSO) or dimethyl acetamide (DMA) prior to freezing. This shows the deleterious effects a diluent and cryoprotectant can have on sperm motility and viability of cockerel semen prior to cryopreservation (Baguio and Capitan, 2008). Researchers have also recorded the sperm motility in fresh semen samples of New Hampshire males to be 73.9±0.2% and the White leghorn 83.2±0.6% respectively (Chalah *et al*., 1999; Bah *et al*., 2001). These sperm motility results are higher than the results obtained in this study (estimated sperm motility), which ranged from 58.8±12.5% to 63.8±13.6%. This may also be due to the time taken before the semen was processed and evaluated and the season of semen collection. Other researchers have reported the percentage of motile sperm to vary between 70.1 ± 0.6% and 67.9 ± 0.5% for cockerels, which is very similar to the findings of this study. Season affects semen

production, so for example the rainy season has been shown to favour the rate of spermatogenesis. The rainy season has also been associated with a high ejaculate volume, sperm concentration and high fertility in poultry (Machebe and Ezekwe, 2005). Cockerels are seasonal breeders and generally produce more semen at the onset of the breeding season (long daylight length) and lower volumes towards the end. Light intensity also affects semen characteristics during the warm and cold season, resulting in lower ejaculate volumes relative to the rainy season, which may be attributed to reduced spermatogenesis and a higher sperm mortality rate. High relative humidity also causes a temporary decrease in sperm production, hence low ejaculate volumes and sperm concentration that could affect sperm motility and fertility (Obidi *et al*., 2008).

The current trial showed that the simple inexpensive method of eosin-nigrosin staining could be used to evaluate cockerel semen quality and as a consequence, estimate the fertilizing capacity of the sperm (although this method still stays subjective). The quantity and quality of fresh semen still stays largely dependent on the individual male (Lukaszewicz and Kruszynski, 2003). The percentage of live sperm recorded was high, ranging between 75.9 ± 33.3% and 80.7 ± 27.9%. Normal morphologically intact sperm constituted between 77.3 ± 17.1% and 84.8 ± 9.2%. The most frequent sperm abnormality recorded in the in this study was in the sperm mid-piece (7.9 to 11%), followed by the sperm head (2.9 to 7.7%). Tselutin *et al.* (1999) reported the number of live sperm without any abnormalities in cockerel semen to vary from 91 to 94%, which is contrary to the results of this study. However, Siudzinska and Lukaszewicz (2008a) recorded 58 to 70% live, morphologically normal sperm and Lukaszewics *et al.* (2008) 70 to 80% live normal sperm, which is again more consistent with the results obtained in this study. The percentage dead sperm recorded during semen collection of 4 poultry breeds ranged between 14 to 27%, which was high, and is more

consistent with the 18 to 24% recorded in this study (Siudzinska & Lukaszewicz, 2008b). The higher number of dead sperm recorded in the study may be attributed to the cold weather, as the study was conducted in winter and it was not easy to keep all the equipment at the recommended temperature to avoid increased sperm mortalities (cold shock) and abnormalities. The morphological sperm defects generally affect the fertility more than the semen motility. Regardless of the semen extender used and male breed, the number of live and normal sperm declined due to the semen storage time before evaluation of semen, as temperature changes affect sperm viability, hence increased sperm abnormalities (Lukaszewicz, 2003). It was also observed that the sperm head and mid-piece defects were higher than the other abnormalities. Similar results have been reported by Alkan *et al*. (2001) and attributed to the chicken sperm cell having a relatively long mid-piece. A number of researchers have reported that the acrosome and the mid-piece are the most sensitive regions in the cockerel's sperm, with the mid-piece being quicker to deteriorate than the other regions. It has also been found that the connecting area between the sperm head and mid-piece of poultry is more sensitive to external factors. The most frequent type of sperm tail defects recorded in the cockerel was bending and folding and the technique of making the stain could contribute to these defects (Alkan *et al*., 2001).

4.5 Conclusions

From the results obtained in this study, the body weight of the different poultry breeds had real effect on cockerel semen production. The White Leghorn breed had the lightest body weight of the four breeds used, but produced a larger ejaculate volume, compared to the heavier Potchefstroom Koekoek. In semen evaluation methods, the human factor is very important and cannot be ignored with differences in results often being related to individual bias and experience (Lukaszewicz *et al*., 2008). Semen quality of cockerels did not differ

much from those reported in other breeds of chickens. It is however important to select males with a higher semen volume, higher sperm concentration and larger number of viable sperm, with few abnormalities, especially for breeding purposes to obtain higher fertilization rates. In this study all exotic breeds recorded higher ejaculate volumes when compared to the local South African breeds.

During AI, the number of sperm per insemination is crucial and cockerel ejaculate semen dilutions can be made depending on the breed semen production performance, as these seem to differ significantly between individuals. It is important to also consider the morphology of the sperm for the different breeds as they also differ significantly. The quality of sperm is to a large extend also determined by the sperm abnormalities. As could be expected, the higher the sperm abnormalities of individual cockerels or breeds, the lower the quality of semen and fertilizing ability of the sperm and hence fertilization rate following AI or natural mating.

When contemplating the processing and cryopreservation of cockerel semen the evaluation of semen quality is of utmost importance. From the semen assessment done in this study, it can be seen that the techniques used for semen evaluation may be relevant to all breeds of chicken and can be used as a tool to estimate the quality of cockerel semen prior to the processing, cryopreservation and AI in an intensive chicken production system.

Chapter 5

Comparative assessment of cryopreserved cockerel semen quality and fertility in four different South Africa chicken breeds

5.1 Introduction

The cryopreservation of poultry (more specifically chicken) semen is an artificial biotechnological technique of reproduction that can be used to increase the diffusion of superior genes and initiate genetic progress, while sustaining and conserving genetic biodiversity. Regardless of the constraints, the approval of an international agreement on biodiversity has aroused new interest in the development of methods of semen freezing in specifically the chicken industry (Blesbois, 2007). Researchers have failed or were slow to develop poultry semen cryopreservation protocols for a long period of time, and this resulted in the loss of valuable alleles (Gill & Barbato, 2001). The increasing importance of artificial insemination (AI) in chicken reproduction (being one of the world's most fast-growing industries) has created much interest to researchers and breeders. Firstly to develop acceptable conditions for liquid (short term) and/or then for frozen (long term) semen storage (Siudzinska & Lukaszewicz, 2008b).

The technique of semen preservation will make it possible to increase the number of hens to be inseminated using AI, while long term semen storage (cryopreservation) at low temperatures will enable the creation of sperm banks of selected cockerels to be used for AI, even long after their death (Latif *et al.,* 2005). Semen cryopreservation in poultry is currently not widely used in conservation programmes, as the success rates are highly dependent on two factors. Firstly, the fertility of the breed and the high variation in successful cryopreservation results obtained between individual birds of the same breed (Blesbois *et al.,* 2008). The main objective of evaluating cockerel semen quality is ultimately to predict the

fertility of the individual male, although these tests used to predict semen quality are often time consuming, labour intensive and unreliable (Parker & McDaniel, 2002).

The fertility and hatchability are interrelated heritable traits and varies between breeds and individuals within breeds. Fertility and hatchability are the most essential determination for producing more chicks from given number of breeding stock within a predetermined period (Islam *et al.,* 2002). Several factors may influence fertility after AI, and these include semen quality, age of the hen, season of the year, body weight and diet, while timing of AI is also important, and is usually performed in the afternoon to minimize the number of hens with hard shelled eggs in the shell gland (Donoghue & Wishart, 2000).

The main objective of this study was to characterize the performance of 4 chicken breeds (Rhode Island Red, Potchefstroom Koekoek, New Hampshire and White Leghorn), regarding the viability, morphology and possible fertilizing ability of cryopreserved semen, post thawing.

5.2 Material and Methods

5.2.1 Semen collection

Semen was collected from 21 cockerels (4 breeds) three times per week for a period of 3 weeks. Semen was collected at ambient temperature in graduated tubes and maintained at 42°C in a warm bath, until microscopically evaluated. Contaminated semen samples (containing blood or fecal material), were discarded. Ejaculate volumes were recorded directly from the graduated semen collection tubes.

5.2.2 Semen processing and cryopreservation

The individual cockerel ejaculates were diluted using the Beltsville Poultry Semen Extender (mBPSE) of Sexton (1977) and modified by the addition of a glucose (instead of fructose) extender fraction A in the ratio of 1:1 (v/v), before freezing. Sperm motility was microscopically evaluated prior to equilibration, with the semen being maintained at room temperature in the collection tube for 20 minutes, and then equilibrated for 1h at 4°C. The semen was again diluted in the ratio of 1:1 (v/v) with BPSE+ 10% DMSO and equilibrated for another hour (4°C) (Han *et al*., 2005). Twenty minutes before semen cryopreservation, dry ice was prepared and 0.2 ml semen pipetted into the indents made on the dry ice. The resulting frozen semen pellets were then plunged directly into the liquid nitrogen (-196°C) for storage (see 3.5 and Plate 3.8 and 3.9).

5.2.3 Evaluation of cryopreserved cockerel semen

For the sperm evaluation after cryopreservation, frozen semen pellets were thawed in a test tube in a water bath at 60˚C, for 2 minutes. The sperm motility was then microscopically evaluated (x40 magnification) immediately after thawing, and the sperm viability (percentage live) determined using an eosin-nigrosin stain (see 3.4). A total of 100 sperm cells were counted per slide, and the results expressed as a percentage live sperm and morphologically normal sperm cells per semen batch.

5.2.4 Artificial Insemination (AI) of the chicken

AI in all hens was performed between 12:00 and 15:00, as most hens have an egg in the oviduct in the morning, thus obstructing the free passage of semen to the ovary. AI was performed using 1.0 ml syringes for the deposition of the semen as described by Sadanand *et al*. (2004). During the insemination process the cloaca was turned inside out and 0.2 ml of the

semen was deposited inside the oviduct. AI was performed for a period of 2 weeks. Both fresh and frozen semen were microscopically evaluated for sperm motility prior to insemination.

For fresh semen AI, hens were inseminated once in a week (Tuesday) immediately after semen collection and evaluation. The semen from each of the Rhode Island Red, Potchefstroom Koekoek, New Hampshire and White Leghorn cockerels was inseminated into 10 (same breed) of the Rhode Island Red, Potchefstroom Koekoek, New Hampshire and White Leghorn hens, respectively. For frozen semen AI, hens (of the same breed) were inseminated twice in a week (Tuesday and Thursday), with pellets for Rhode Island Red, Potchefstroom Koekoek, New Hampshire and White Leghorn cockerels being thawed at the time of insemination, and immediately inseminated to the 10 hens of their respective breed.

5.2.5 Collection and incubation of the eggs

The day of artificial insemination (AI) was recorded as day 0 of fertilization for each hen. After a single insemination with both fresh and frozen semen, eggs were collected daily from the 2nd day post insemination, for a period of 7 days after AI. This was taken as the first day of the production of fertile eggs. The eggs were labeled in order to identify the different breeds (Rhode Island Red, Potchefstroom Koekoek, New Hampshire and White Leghorn), type of semen used (fresh or frozen) and the day of collection. All eggs were collected in the afternoon (between 13:00 and 15:00) and stored at room temperature (20 - 25˚C), prior to incubation for 18 days, at 37˚C. Cracked and dirty eggs were not incubated and discarded.

On the 18th day of incubation all (973) the eggs were candled, to identify the fertile eggs (Islam *et al.,* 2002). Only fertile eggs were then further incubated in the hatchery for another period of 3 days (total 21 days). The eggs that did not hatch were cracked open, to determine

the possible cause of infertility or non-hatching. The fertility, number of hatched and dead chicks were counted and recorded for all the 4 breeds including treatments (fresh and frozen AI). The following temperatures and humidity was maintained during incubation period:

a) Setting temperature (37˚C) of eggs up to 18 days of incubation.

b) Hatching temperature (37˚C) of chicks from 18-21 days of incubation.

c) Setting Relative humidity: 60% up to 18th day of incubation (Banks, 1979).

d) Hatching Relative humidity: 60%from 18-21 days of incubation (Banks, 1979).

5.3 Results

The cryopreservation process resulted in a significant decrease in the total live sperm, when compared to that of the fresh cockerel semen as set out in Table 4.2 (Chapter 4). The differences between the 4 chicken breeds regarding the morphology of the frozen-thawed sperm were not significant. In all frozen-thawed ejaculates, the percentage total live sperm recorded ranged between 37.4 and 42.3%, with the percentage of dead sperm ranging between 57.7 and 62.4% as shown in Table 5.1 and 5.2 respectively. The number of sperm abnormalities specifically (mid-piece and tail abnormalities) increased to a lesser degree following cryopreservation (Table 5.2), while in all 4 cockerel breeds there was an increase in the number of sperm head abnormalities recorded, due to the freezing process (ranging from 17.3 to 22.5%). In the fresh semen samples, the sperm motility of the cockerel semen was higher and ranged between 3.6 and 3.9 (out of a possible score of 5), while in the frozen-thawed samples, the sperm motility was slow to moderate (score of 1.9 to 2.1). The ejaculate volume of the 4 chicken breeds varied from 0.4 to 0.5ml (spring), compared to volumes recorded in winter (0.3 to 0.4ml) of the current study. This volume was slightly lower,

suggesting a possible seasonal effect in the semen production of the cockerel, irrespective of the breed.

Table 5.1 The mean (±SD) seminal characteristics of cockerel semen collected from 4 different chicken breeds before and following cryopreservation.

	Fresh semen		**Cryopreserved semen**		
Breed	**Ejaculate Vol (ml)**	**Sperm Motility (0-5)**	**Post/thaw Motility (0-5)**	**No. Pellets collected**	**Live Sperm (%)**
Rhode Island Red	0.5^{a} ±0.2	3.9^{a} ±0.3	2.1^{a} ±0.6	6.0^{a} ±1.7^{a}	41.4^{a} ±11.8
Potchefstroom Koekoek	0.4^{b} ±0.1	3.9^{a} ±0.4	2.1^{a} ±0.6	4.8^{ab} ±2.9	42.3^{a} ±12.1
New Hampshire	0.5^{ab} ±0.2	3.6^{a} ±0.5	1.9^{a} ±0.6	4.5^{b} ±1.7^{b}	37.4^{a} ±10.4
White Leghorn	0.4^{b} ±0.1	3.7^{a} ±0.5	2.0^{a} ±0.6	4.5^{b} ±1.4^{b}	38.3^{a} ±6.8

[a-c:] Means within a column with different superscripts differ significantly (P<0.05).

Table 5.2 Mean (±SD) sperm abnormalities of cryopreserved cockerel semen from 4 chicken breeds, immediately after thawing.

Breed	**Dead Sperm (%)**	**Head Abnormalities (%)**	**Mid-piece Abnormalities (%)**	**Tail Abnormalities (%)**	**Total Sperm Abnormalities (%)**
Rhode Island Red	58.6^{a} ±11.8	20.0^{a} ±11.5	7.9^{a} ±3.8	2.0^{a} ±2.8	30.0^{a} ±11.7
Potchefstroom Koekoek	57.7^{a} ±12.1	17.3^{a} ±11.4	9.1^{a} ±5.4	1.2^{a} ±1.6	28.3^{a} ±11.9
New Hampshire	62.4^{a} ±10.8	22.5^{a} ±10.3	4.3^{a} ±1.4	1.6^{a} ±1.9	28.4^{a} ±14.3
White Leghorn	60.9^{a} ±6.2	19.1^{a} ±7.6	9.9^{a} ±6.7	0.5^{a} ±0.9	30.2^{a} ±9.2

[a-c:] Means within a column with different superscripts differ significantly (P<0.05).

The fertility rate, percentage infertile eggs, hatchability and percentage dead chicks in the shell of hens inseminated with fresh semen are set out in Table 5.3. The fertility rate recorded

was highest in the Rhode Island Red and White Leghorn, followed by the Potchefstroom Koekoek and lastly the New Hampshire. However, hatchability of the eggs following AI with fresh semen was 78.4, 67.8, 62.1 and 43.3%, respectively (Table 5.3). The percentage dead chicks in the shell was again highest in the White Leghorn breed (14.4%), followed by Potchefstroom Koekoek (8.9%), New Hampshire (6.7%), and Rhode Island Red (6.0%) respectively.

Table 5.3 Fertility rate and hatchability obtained following AI with fresh semen in different chicken breeds

Breeds	**Fertile eggs (%)**	**Infertile eggs (%)**	**% Hatching of fertile eggs**	**Chicks dead in shell (%)**
Rhode Island Red	84.5	15.5	78.4	6.0
Potchefstroom Koekoek	71.0	29.0	62.1	8.9
New Hampshire	50.0	50.0	43.3	6.7
White Leghorn	82.2	17.8	67.8	14.4

Similarly the different fertility rates and hatchability traits of the Rhode Island Red, Potchefstroom Koekoek, New Hampshire and White Leghorn following AI with cryopreserved semen are set out in Table 5.4. Following the detrimental morphological changes induced in the sperm cell following cryopreservation, the low fertility rate recorded was highest in the White Leghorn, followed by the Rhode Island Red, Potchefstroom Koekoek and New Hampshire (27.3, 12.8, 12.7 and 2.7% respectively) and these were not in the same sequence as following fresh semen AI. The percentage hatchability of the eggs following successful fertilization with cryopreserved semen was highest in the White Leghorn and Rhode Island Red, followed by the Potchefstroom Koekoek and lastly the New

Hampshire (13.6, 12.8, 9.7 and 2.7% respectively). When using fresh semen AI, the percentage of chicks dead in the shell of the White Leghorn (17.6%), New Hampshire (13.3%), Potchefstroom Koekoek (12.5%) and Rhode Island Red (7.1%) were much higher than the 3.0% recorded in the Potchefstroom Koekoek and 13.6% in the White Leghorn following the use of cryopreserved semen.

Table 5.4 Fertility rate and hatchability following AI with frozen-thawed semen in different chicken breeds

Breeds	**Fertile eggs (%)**	**Infertile eggs (%)**	**Hatching of fertile eggs (%)**	**Chicks dead in shell (%)**
Rhode Island Red	12.8	87.2	12.8	0.0
Potchefstroom Koekoek	12.7	87.3	9.7	3.0
New Hampshire	2.7	97.3	2.7	0.0
White Leghorn	27.3	72.7	13.6	13.6

5.4 Discussion

Species-specific differences in the ability of cockerel sperm to withstand the various stresses caused by cryopreservation have long been identified as a major problem in the preservation of animal genetic material and to preserve biodiversity (Blesbois *et al.,* 2005, Siudzinska and Lukaszewicz, 2008a). The cryopreservation procedure for cockerel semen, tested in this trial induced highly significant and detrimental changes in semen quality, which could be expressed as a decrease in sperm viability and morphological integrity of the sperm, in all the breeds (Rhode Island Red, Potchefstroom Koekoek, New Hampshire and White Leghorn). A

particularly prominent increase in the percentage dead sperm was recorded in all birds and a difference between the breeds, regarding the ability to freeze successfully was also observed.

Han *et al.* (2005) reported the degree of cytotoxicity of the cryoprotectant DMSO to be influenced by the type of extender used. In that specific experiment, 10% DMSO gave the highest sperm motility (70.8%) when using potassium citrate, sodium glutamate, potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium dihydrogen phosphate and inositol as components of the extender. The lowest sperm motility of 9.2% on the other hand was recorded with the use of a specific extender (with magnesium acetate, potassium citrate, sodium glutamate, lactose, glycine, mannite and Tris as components of the extender). Therefore it may be possible that the appropriate combination of components of a specific extender reduces the cytotoxicity of DMSO and yielded a higher percentage motile sperm cells. Blesbois *et al.* (1999) recorded the sperm motility to decrease from 87.5 to 46% and the viable and morphologically normal cells to decrease from 84 to 48% following cryopreservation. Tai *et al.* (2001) recorded a sperm motility of 2.0 ± 2.5 and 7.3 ± 4.7 % live sperm, when using 4% DMSO in the cryopreservation extender. According to the results of the different researchers, the variation in the sperm motility of frozen-thawed semen may be attributed to the cryopreservation technique used, the composition of the diluent and type of cryoprotectant and the percentage of cryoprotectant used (Tai *et al*., 2001; Han *et al.,* 2005).

Generally the ability of sperm to survive following cryopreservation is different in different chicken breeds. In the current study, a higher percentage of viable sperm (percentage live) in the cryopreserved semen (although not significant) was recorded in the Potchefstroom Koekoek cockerels, followed by the Rhode Island Red, White Leghorn and New Hampshire respectively. The importance of initial fresh semen quality is crucial regarding the final success achieved following cryopreservation and aids to predict the suitability of the sperm in

withstanding the hazards of the cryopreservation and the thawing processes (Lukaszewicz, 2002; Blesbois *et al.,* 2007, 2008). The quality of fresh semen in certain breeds and individual cockerels was generally low and on the basis of the results recorded, it can be stated that significant differences in semen freezability exists between the Rhode Island Red, Potchefstroom Koekoek, New Hampshire and White Leghorn breeds. Variation even exists between individuals of the same breed.

The post-thawing sperm survival rate of the New Hampshire semen was generally much lower than the fresh semen quality recorded earlier, and most of the individual ejaculates did not survive cryopreservation. The process was thus deleterious, hence the small number of frozen semen pellets obtained following processing in this breed. When taking into account the differences between the fresh and frozen-thawed semen, it is evident that the number of live sperm cells, which survive the cryopreservation process are dependant of the initial fresh semen quality used. The decrease in the semen quality caused by cryopreservation also accentuates any sperm damage or abnormalities that were present in the fresh ejaculate. Poor fresh semen quality (sperm motility and abnormalities) produces a resultant very poor frozen semen product (Lukaszewicz and Kruszynski, 2003; Blesbois *et al.,* 2007, 2008). It also appears as if the number of sperm that survive the cryopreservation process does not only depend on the fresh semen quality, as judged by the sperm morphology evaluated under a light microscope, but also the significance of choosing a suitable cryoprotective medium (diluent and cryoprotectant), being crucial.

Chalah *et al.* (1999) reported that, depending on the cryopreservation technique used, one-third to half of the total number of sperm survive the deleterious effects of the cryopreservation procedure. Subsequently when semen was frozen in pellets using DMA as a cryoprotectant only 19% viable sperm were recorded in thawed cockerel semen, while Tai *et*

al. (2001) recorded a 7.3% sperm survival rate after using 4% DMSO. This is lower than the post thawing motility recorded in the present study. Seigneurin and Blesbois (1995) recorded approximately 34 to 48% normal and viable sperm while Lukaszewicz (2002) recorded a total percentage live sperm of 68% normal and viable sperm which is again higher than the results obtained in this study. Seigneurin and Blesbois (1995) also recorded a 33 to 53% sperm survival rate of frozen-thawed chicken semen which again is in agreement with the results recorded in the current study (38.3 to 42.3%). Blesbois *et al.* (2005) also recorded significantly higher percentages of viable and morphologically normal sperm in cryopreserved chicken semen (32%), compared to cryopreserved semen of turkey (25%).

With no reference to breed, the overall cryopreservation of cockerel semen as applied in this study generally caused a significant and detrimental decrease in changes to the semen quality. These were manifested mainly by the high proportion of dead sperm and sperm with head defects or abnormalities. The total number of dead sperm recorded varied between 57.7 and 62.4% in the current study, which is similar to the results recorded by Seigneurin and Blesbois (1995) of 52.4 to 66.8%. The cockerel semen motility recorded by Tai *et al.* (2001) following cryopreservation was 2.0, which is again similar to the results obtained in the present study (1.9 to 2.1).

The sperm activity at the site of fertilization, especially following cryopreservation in the chicken can induce a reduction in the total number of chicks hatched. This is mostly due to the fact that relatively few sperm survive the cryopreservation process satisfactory, to fertilize the egg. This lower hatchability is associated with a reduction in fertility, as well as an increase in the embryonic mortality, which is common in older hens or a flock experiencing infrequent mating or AI activity (Bramwell, 2002). So for example the motility of cockerel sperm recorded immediately after thawing was less, when compared to a fresh semen sample.

The sperm motility, after cryopreservation in the mBPSE containing 10% DMSO, however, was not a good example of a cryodiluent for acceptable sperm fertilizing ability. Although sperm motility was recorded as an indicator of sperm viability, fertilization is not always guaranteed. This sperm motility in the frozen-thawed semen population did not appear to be fully functional, or be able to fertilize, as the AI of motile thawed sperm does not always result in an acceptable percentage of fertile eggs being produced in all breeds. However, acceptable fertility results were obtained following AI with fresh semen (Table 5.3), serving as an indicator that the AI and even the semen collection technique used, were acceptable.

Generally the fertility rate recorded was high in the White Leghorn (layer), compared to the dual purpose breeds like the Rhode Island Red, Potchefstroom Koekoek, and New Hampshire. Here again the hatchability was relatively lower in the White Leghorn, which could possibly be attributed to the large size of the eggs laid. These observations are in agreement with the findings of Abiola *et al.* (2008), who reported a lower hatchability of large eggs when incubating eggs of varying size. Other factors that affect hatchability include egg fertility and embryonic mortality (Fairchild *et al.,* 2002). Some of the causes of embryonic mortality reported being a prolonged egg storage time, the season of the year, nutritional status, egg size, age of the breeders and technical incubation problems (Fasenko *et al*., 2001; Wilson, 1997; Fairchild *et al*., 2002). In the current study, the highest fertility when using fresh semen AI was recorded in the Rhode Island Red (84.5%) breed, followed by the White Leghorn (82.2%), Potchefstroom Koekoek (71%) and lowest in the New Hampshire (50%). These results are in agreement with findings of Islam *et al*. (2002), recording a 94.0% (White Leghorn) and 90.0% fertilization rate (Rhode Island Red), respectively. This is again an indication that the AI technique used was satisfactory, furthermore, the hatchability of the eggs set, concur with that of Molekwa and Umesiobi (2009), who found significant

differences in the hatchability of fertile eggs, between breeds. However, the fertility and hatchability of the Potchefstroom Koekoek, which was recorded as 84.0 and 74.3% respectively, was higher than that obtained in the present study. The fertility rates obtained following fresh semen AI in the current work are also in agreement with Petek and Dikmen (2006), who recorded values ranging between 61.8 and 85.4%.

In the domestic fowl, it has been found that the sperm numbers, type of hens (broiler or layer types) and age may affect the in vivo storage of the sperm and subsequently, the fertility of the eggs (Tabatabaei *et al*., 2009). It could be expected that the fertilizing capacity of frozen-thawed cockerel semen to be much lower following exposure of the sperm cell to the freezing process, with subsequent sperm damage and hence a lower fertilizing capacity. There are many factors that also affect the post thawing fertilizing capacity. These include the ultrastructural, biochemical, functional damage, cold shock, cooling rate, diluent composition and osmotic stress (Holt, 2000; Watson, 2000; Dorado *et al*., 2007). Semen cryopreservation techniques for the fowl render a fair post-thaw sperm survival of up to 60%. However, there is a striking variation (and thus room for improvement) between studies in the reported percentages of fertilized eggs, ranging from 9 to 91% (Hiemstra *et al.,* 2005).

The fertility of the eggs laid between the 4 breeds studied was different, and this may be attributed to several reasons. Different fertility results have been obtained after AI with frozen semen using different genetic lines, which may imply that the differences in tolerance of the cryopreservation treatment displayed by the sperm may be attributable to genetic causes. This could imply that individual cockerels must be selected according to their ability of their semen to freeze successfully. Also the sperm cell is composed of varying amounts of protein, which may influence the absorption of the cryoprotectants, and other sperm cell again display higher oxygen consumption. This observation may be interpreted as indicating

that the sperm cell organelles of certain cockerels are more resistant to the damage caused by freezing as such (Bellagamba *et al*., 1993). The occasional higher fertility rates obtained using frozen-thawed semen in poultry may seem surprising, but could be attributed to the higher dose levels of sperm and higher frequency of AI performed, when using frozen-thawed semen (Hiemstra *et al.,* 2005; Blesbois *et al.,* 2007, 2008).

The fertility rates obtained in the present study were found to range between 2.7 and 27.3%, with a hatchability range of 50 to 100%. Blesbois *et al.* (2007) obtained 2.8% where glycerol was used as a cryoprotectant, and 30.4% where DMA was used as a cryoprotectant. A 0 to 62% hatchability was also recorded, which could suggest that the semen cryopreservation technique used in the current study was readily acceptable.

5.5 Conclusion

The cryopreservation of cockerel semen, along with artificial insemination, could make an important contribution to the improvement of genetic progress in poultry production. The initial fresh semen quality of the New Hampshire breed was recorded to be low, compared to the other three breeds of chicken evaluated. Generally semen cryopreservation subsequently induced higher sperm mortalities in the New Hampshire, which ultimately led to lower survival rates after cryopreservation and thawing. There was no significant difference recorded in the percentage live sperm between the 4 breeds evaluated. Among the numerous factors that contribute to the variation in the sperm motility induced following semen cryopreservation, cockerel breed and the potential semen freezability of the individual bird is very important. There still exists a necessity to develop an ideal cryopreservation method, which would allow for the long term storage of cockerel semen for decades in liquid nitrogen (-196˚C), with the minimum loss in sperm viability and fertilizing capacity.

The quality of the semen prior to cryopreservation would seem acceptable, although the cockerel sperm failed to give satisfactory fertility rates following AI with the cryopreserved semen. The differences in the freezing ability between breeds and even individual birds make interpretation of the data difficult. The importance of the quality of the fresh semen prior to freezing cannot be overestimated, with a variety of external factors (cryopreservation technique, diluent, thawing procedure, etc) that can play a role in the final fertilization rate obtained following cryopreservation and thawing. The advantages of semen cryopreservation in the poultry industry hold great promise in accelerating the genetic progress and the storage of superior genetic material for extended periods of time. The fact that normal healthy chicks were induced following the cryopreservation of cockerel semen, although low, should be enough motivation to warrant further studies to increase the efficiency of cockerel semen cryopreservation in the chicken industry. The technique of semen collection and AI (fresh and frozen semen) would seem acceptable. This study was one of the first controlled cryopreservation trials done in South Africa. The fact that some success was obtained is encouraging, although much more work in refining the technique has to be done.

Chapter 6

General Conclusions and Recommendations

6.1 General Conclusions

Cryopreservation of chicken semen, along with artificial insemination could make an important contribution to the poultry industry, especially regarding genetic improvement. However, suitable methods, specific to a chicken breed have to be developed for successful semen cryopreservation. There have been continued individual variations recorded in sperm freezability, which are genetically and environmentally determined and may be related to processes that affect spermatogenesis. It is possible to distinguish groups of males within a specie or within a breed on the basis of a consistent positive response to a specific semen cryopreservation protocol. Moreover, each procedure has its own inherent particular variables, such as type of diluent, dilution rate, cooling rate, nature and percentage of cryoprotectant, freezing procedures, choice of packaging (semen straws or pellets) and thawing procedures. Together these factors could contribute to a better and more acceptable sperm survival rate following cryopreservation.

Characterization of cockerel semen before cryopreservation is an important initial starting point, as it is essential to select cockerels with an acceptable motility, a higher ejaculate volume and total sperm concentration to obtain higher fertility rates. The cockerel semen variation was recorded to be significant regarding ejaculate volume, sperm concentration (semen density) and the percentage normal sperm in this study. The semen parameters of the indigenous cockerels all recorded comparable values with that of the more exotic breeds, following microscopic evaluation. This means that the indigenous cockerel breeds studied can compete favourably with exotic breeds in a chicken AI programme. However, it is

important to select cockerels with a higher ejaculate volume and sperm concentration for breeding purposes, to obtain higher fertility rates.

Although the present study indicates inter-breed differences in sperm freezability to occur, it can be concluded that the simple, relatively inexpensive, and easily adaptable and applied field condition techniques of semen cryopreservation in the pellet form, can be used to preserve the sperm of poultry breeds and facilitate gene exchange and genetic process in breeds. In all poultry species, this must encompass a better basic understanding of the variation in sustainability of freezing semen in different species, including individual and breed differences. All this must be done to obtain acceptable frozen semen samples needed to further genetic progress and the long term gamete preservation for superior chicken traits. However, it must be emphasized that the susceptibility of sperm cryopreservation collected from different breeds and individuals was highly variable. The ultimate important consideration is the immediate need for the preservation of semen from certain poultry lines, currently at risk. Developing alternative cryopreservation protocols for poultry semen is ultimately a lengthy process, but needs attention. The fact that some success has been obtained in the collection, semen evaluation, dilution and cryopreservation of cockerel semen, is encouraging. The further use of AI (fresh or frozen) further emphasizes the importance of this current research.

Numerous studies have shown a variation to exist in the fertility of cockerels, within the same breed, and even in the same animal, while the fertilizing ability of sperm do not remain constant throughout the year (seasonality). It is important to recognize and evaluate the factors that affect the quality of semen, as an increase for example, in the percentage of abnormal sperm can cause a subsequent reduction in fertility. It is understood that the fertility level can also be affected by factors related to the male in two ways. Firstly by inherent or

physiological factors affecting the quality of sperm collected artificially. Secondly other factors associated with the procedure of AI and the female, which may influence the fertility, e.g. the number of sperm inseminated, the site of deposition of semen in the oviduct, timing of insemination and the changing of the oviductal environmental factors associated with the reproductive status of the female.

Fertility and hatchability of the eggs are further essential in the determination of producing a large number of live chicks from a set number of breeding stock, within a predetermined period of time. The fertility and the hatchability performance of eggs also depend on a number of factors such as genetic, physiological, social and environmental effects. Egg hatchability and embryonic mortality can further be affected by factors which include prolonged egg storage, poor storage conditions, season of the year, nutritional status, egg size, age of the breeders and incubation shortcomings. In general, the results of this study indicate that fertile eggs and chicks can be produced by inseminating hens with frozen-thawed semen cryopreserved in mBPSE extender containing 10% DMSO, irrespective of the breed. Considering the overall fertility, hatchability and the number of live chicks recorded in this study using frozen-thawed semen, although low, it should serve as a motivation to increase the efficiency of cockerel semen cryopreservation to benefit the chicken industry.

6.2 Recommendations

The eosin-nigrosin method for determining the percentage live and dead sperm and the structural morphology of the sperm can be recommended for use in farms where chicken flocks are reproduced using AI with fresh semen, and even semen evaluation prior to cryopreservation. There should be adequate numbers of males available for more specific selection of cockerels regarding the semen freezability of the individual. To increase sperm

viability and the other parameters following cryopreservation, more research is necessary regarding more effective extenders (diluents and cryoprotectants) specific to a certain breed.

Abstract

Characterization and cryopreservation of semen of four South African indigenous poultry breeds

by

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The aim of the study was to characterize and evaluate the quality of fresh semen of 4 breeds of chicken and the susceptibility of cockerel semen to a cryopreservation protocol, assessed microscopically for sperm motility and morphology and ultimately fertilizing ability following AI. The differences between breeds were determined by comparing the fertilizing ability and hatchability of fresh and frozen-thawed semen. The study was carried out at Glen Agricultural Development Institute and at the University of the Free State. Four chicken breeds, namely the Rhode Island Red, Potchefstroom Koekoek, New Hampshire and White

Leghorn, were used. Qualitative characterization of the semen was performed in 28 cockerels (7 per breed). Semen was collected using the massage technique twice a week in the first trial. The eosin-nigrosin staining technique was used to microscopically evaluate the morphology of the sperm from the different breeds. The fresh semen parameters evaluated were ejaculate volume, semen colour, semen pH, sperm concentration, the percentage live and dead sperm, sperm motility and the abnormalities of the sperm. The percentage live and dead sperm, sperm motility and abnormalities were also evaluated for the frozen-thawed cockerel semen.

During the second phase of the study, semen was collected 3 times per week from the same cockerels. Semen was frozen using a fast–freezing procedure on dry ice, with 10% DMSO as the cryoprotectant. AI was performed on 4 different breeds of hens (20 hens per breed) (Rhode Island Red, Potchefstroom Koekoek, New Hampshire and White Leghorn), using fresh semen (control) and frozen-thawed semen. During AI of each breed, 10 hens were inseminated with fresh and the remaining 10 hens with frozen-thawed semen.

The sperm characteristics of the semen samples of the 4 breeds recorded were ejaculate volume, ranging from 0.3±0.1 to 0.4±0.1ml, semen pH of 7.6±0.4 to 7.7±0.3, sperm motility (scale of 0-5) 2.8±0.8 to 3.1±0.9, estimated sperm motility 58.8±12.5 to 63.8±13.6%, ejaculate concentration ($x10^9$ sperm/ml) of 320.4±286.5 to 748.5±475.3, percentage live sperm 75.6±29.1 to 81.5±26.8%, and the percentage dead sperm 18.6±26.8 to 24.4±29.1% respectively, with the percentage normal sperm ranging between 77.3±17.1 and 84.8±9.0%. Head, mid-piece, tail and other sperm abnormalities of the fresh semen of the 4 breeds ranged from 2.9±3.3 to 7.7±9.6%, 7.9±5.2 to 11.0±7.0%, 0.4±0.8 to 1.9±3.0% and 0.6±0.9 to 1.5±1.9%, respectively. Semen samples were frozen in pellet form on a block of dry ice, by pipetting into the indentations on the surface of the ice. The frozen cockerel pellets were

thawed following cryopreservation, by being placed into a test tube in a water bath (60˚C), and the tube shaken continuously until complete thawing of the pellet. During the time of semen cryopreservation, a decrease in the number of live, morphologically normal sperm, and increase in the percentage dead sperm and sperm with abnormalities were recorded. The freeze-thawing process caused a significant ($P<0.05$) decrease in the percentage live sperm and the sperm motility, ranging between 37.4±10.4 and 42.3±12.1% and 3.6±0.5 and 3.9±0.3 respectively. A consequent increase in the percentage of dead sperm (between 57.7±12.1 and 62.4±10.8%) was also recorded. The sperm abnormalities regarding sperm head abnormalities ranged between 17.3±3.8 to 22.5±10.3%, the mid-piece abnormalities 7.9±3.8 to 10.4±2.0% and the tail abnormalities between 0.5±0.9 to 2.0±2.4% respectively for the thawed semen. Frozen-thawed semen was thawed in a water-bath 60˚C and hens were inseminated twice per week using the frozen-thawed semen, and once a week with fresh semen for a total period of two weeks. Data for the two trials were analyzed using the ANOVA and the Tukey's Studentized Range (HSD) test for repeated measures (SAS system General Linear Models Procedure).

A total of 973 eggs, from all breeds of chicken namely the Rhode Island Red, Potchefstroom Koekoek, New Hampshire and White Leghorn were collected following AI with fresh and frozen semen from individually caged hens. Eggs were collected, incubated and hatched to check if fertile and normal chicks could be produced from frozen-thawed cockerel semen. The difference in fertility and hatchability of the hens of the different breeds were compared and found to be highest in Rhode Island Red, White Leghorn, and Potchefstroom Koekoek respectively and lowest in New Hampshire using fresh semen. When using frozen-thawed semen, the sequence of fertility performance was the White Leghorn, Rhode Island Red, Potchefstroom Koekoek and New Hampshire, respectively. The effect of the numbers of

sperm per AI dose on fertility, age at embryonic death, and hatchability of fertile eggs were also evaluated. Low numbers of sperm per AI in the hens resulted in a decrease in the total number of chicks hatched. The lowest fertility rate recorded was in the New Hampshire (2.7%), when using frozen-thawed semen to inseminate the hens. This may be attributed to the low numbers of sperm inseminated per AI dose. Egg hatchability of the fertile eggs was high in the White Leghorn (13.6%), Rhode Island Red (12.8 %), Potchefstroom Koekoek (9.7 %) and low in New Hampshire (2.7%) respectively, which could possibly be attributed to the egg size. Medium sized eggs were preferable for setting, in order to obtain an acceptable hatch, as they generally hatch better than the larger eggs. The results recorded for fertility and hatchability in the control group (fresh semen), was similar to the results recorded by other researchers, showing that the AI method used was acceptable. There still exists a necessity to develop an ideal cryopreservation method (diluent and freezing procedure), which would allow for acceptable long term storage of cockerel semen in liquid nitrogen (-196˚C) for future use and export with minimum loss regarding sperm viability and fertilizing capacity.

List of References

Abiola, S.S., Meshioye, O.O., Oyerinde, B.O. & Bamgbose, M.A., 2008. Effect of egg size on hatchability of broiler chicks. Arch. de Zoot. 57, 83-86.

Adeyemo, G.O., Longe O. G. & Adejumo D. O., 2007. The reproduction performance of breeder cocks fed cottonseed cake-based diets. Int. J. Poult. Sci. 6. 140-144.

Alkan, S., Baran, A., Ozdas, B, O. & Evecen, M., 2001. Morphological defects in turkey semen. Tur. J. Vet. Anim. Sci. 26, 1087-1092

Anderson, J., 2001. The semen of animals and its use for artificial insemination. Greenworld publishers India.

ARC. 2006. The indigenous poultry breeds of SA.

Ashraf, M., Mahmood, S., Khan, M.S. & Ahmad F., 2003. Productive Behavior of Lyallpur Silver Black and Rhode Island Red Breeds of Poultry. Int. J. Agric. Biol. 3, 384-387.

Ayo, J.O. & Sinkalu, V.O., 2007. Effects of ascorbic on diurnal variations in rectal temperature of shaver brown pullets during the hot dry season. Int. J. Poult. Sci. 6, 642-646.

Baguio, S.S. & Capitan, S.S., 2008. Motility, livability and fertility of cock spermatozoa as influenced by day of collection, diluent and cryopreservation. J. Vet. Med. 45, 109-117.

Bah, G.S., Chaughari, S.U.R. & Al-Amin, J.D., 2001. Semen characteristics of local breeder cocks in the Sahel region of Nigeria. Revue Elev. Med. Vet. Pays trop. 54, 153-158.

Banks, S., 1979. The complete handbook of poultry keeping. London, Word lock Limited.

Bearden, H.J., Fuquay, J.W. & Willard, S.T., 2004. Applied Animal Reproduction. Pearson Education, Inc. New Jersey. 6th edition.

Bellagamba, F., Cerolin, S. & Cavalchini, L.G., 1993. Cryopreservation of poultry semen. Wld Poult. Sci. J. 49, 157-162.

Blanco, M.J., Gee, G., Wildt, D.E. & Donoghue, A.M., 2000. Species variation in osmotic, Cryoprotectant, and cooling rate tolerance in poultry, eagle, and peregrine spermatozoa. Biol. Reprod. 63, 1164-1171.

Blesbois, E., Grasseau, I. & Hermier, D., 1999. Changes in lipid content of fowl spermatozoa after liquid storage at 2 to 5˚C. Theriogenology, 52, 325-334.

Blesbois, E., Grasseau, I. & Seigneurin, F., 2005. Membrane fluidity and the ability of domestic bird spermatozoa to survive cryopreservation. Reprod. 129, 371-378.

Blesbois, E., 2007. Current status in avian semen cryopreservation. Wld. Poult. Sci. J. 63, 213-222.

Blesbois, E., Seigneurin, F., Grasseau, I., Limouzin, C., Besnard, J., Gourichon, D., Coquuerelle, G., Rault, P. & Tixier-Boichard., M., 2007. Semen cryopreservation for ex situ management of genetic diversity in chicken: creation of the French avian cryobanks. Poult. Sci. 86, 555-564.

Blesbois, E., Grasseau, I., Seigneurin, F., Mignon-Grasteau, S., Saint Jalme, M. & Mialon-Richard, M.M., 2008. Predictors of success of semen cryopreservation in chickens. Theriogenology, 69, 252-261.

Bootwalla, S.M. & Miles, R.D., 1992. Developments of diluents for domestic fowl semen. Wld. Poult. Sci. J. 48, 121-126.

Bramwell, K. R., 2002. Fertility and embryonic mortality in breeders. Avian advice University of Arkansas. Summer, 4, http://poultryscience.uark.edu/pdfs/

Branckaert, R.D.S. & Gueye, E.F., 1995. FAO's programme for support to family poultry production. Anim. Prod. Anim. Hlth. FAO.

Burrows, W,H. & Quinn, J.P., 1937.the collection of spermatozoa from the domestic fowl and turkey. Poult. Sci. 24, 19-24.

Chalah, T., Seigneurin, F., Blesbois, E. & Brillard, J.P., 1999. In vitro comparison of fowl sperm viability in ejaculates frozen by three different techniques and relationship with subsequent fertility in vivo. Cryobiol. 39, 185-191.

Christensen, V.L., 2001. Factors associated with early embryonic mortality. Wld. Poult. Sci. 57, 359-372.

Chulhong, P. & Chapman, F.A., 2005. An Extender Solution for the Short-Term Storage of Sturgeon Semen. N. A. J. Aquaculture, 67, 52-57

Cole, H.H. and Cupps, P.T., 1977. Reproduction in domestic animals. 3rd ed. New York. Academic press.

Culty, M., Luo, L., Yao, Z., Chen, H., Papadopoulos, V. & Zirkin, B., 2002. Cholesterol transport, peripheral benzodiazepine receptor, and steroidogenesis in aging Leydig cells. J. Androl. 23, 439–447.

Didion, B.A., 2008. Computer-assisted semen analysis and its utility for profiling boar semen samples. Theriogenology, 70, 1374-1376.

Das, S.K., 2002. Effects of feeding on semen production in native cock in Bangladesh. J. Biol. Sci. 2, 810-811.

Donoghue, A. M. & Wishart, G.J., 2000. Storage of poultry semen. Anim. Reprod. Sci. 62, 213-232.

Dorado, J., Rodri`guez, I. & Hidalgo, M., 2007. Cryopreservation of goat spermatozoa: comparison of two freezing extenders based on post-thaw sperm quality and fertility rates after artificial insemination. Theriogenology, 68, 168-177.

Dumpala, P.R., Parker, H.M. & McDaniel, C.D., 2006. The effect of semen storage temperature and diluent type on the sperm quality index of broiler breeder semen. Poult. Sci. 5, 838-845.

Etches, R.J., 1996. Reproduction in poultry. 1st Ed. CAB International, Cambridge, UK.

Fairchild, B.D., Christensen, V.L., Grimes, J.L., Wineland, M.J. & Bagley, L.G., 2002. Hen age relationship with embryonic mortality and fertility in commercial turkeys. J. Appl. Poult. Res. 11, 260-265.

Fasenko, G.M., Robinson, F.E., Whelan, A.I., Kremeniuk, K.M. & Walker, J.A., 2001. Prestorage incubation of long-term stored broiler breeder eggs: 1. Effects on hatchability. Poult. Sci. 80, 1406-1411.

Froman, D.P., Feltmann A.J., Rhoads, M.L. & Kirby, J.D., 1999. Sperm mobility: a primary determinant of fertility in the domestic fowl (Gallus domesticus). Biol. Reprod. 61, 400-405.

Froman, D.P. & Feltmann, A.J., 2005. Fowl (Gallus domesticus) sperm motility depends upon mitochondrial calcium cycling driven by extracellular sodium. Biol. Reprod. 72, 97-101.

Froman, D.P., 2006. Application of the sperm mobility assay to primary breeder stock. Appl. Poult. Res. 15, 280-286.

Galal, A., 2007. Predicting Semen Attributes of Naked Neck and Normally Feathered Male Chickens from Live Performance Traits. Int. J. Poult. Sci. 1, 36-42.

Gill, S.P. & Barbato, G.F., 2001. Cryopreservation of sperm: influence of genotypes and methodologies. Dept. Poult. Sci. Pennsylvania State University.

http://www.fass.org/fass01/pdfs/Gill.pdf.

Gomendio, M. & Roldan, E.R., 2008. Implications of diversity in sperm size and function for sperm competition and fertility. Int. J. Dev. Biol. 52, 439-447.

Gordon, I., 2005. Reproductive technologies in farm animals. CABI Publishing UK.

Hafez, B. & Hafez, E.S.E., 2000. Reproduction in Farm Animals. 7th ed. New York. Lippincott Williams & Wilkins, USA.

Hafez, E.S.E ., 1974. Reproduction in Farm Animals. 3rd ed. Lea & Febiger, USA.

Han, X.F., Niu, Z.Y., Liu, F.Z. & Yang, C.S., 2005. Effects of diluents, cryoprotectants, equilibration time and thawing temperature on cryopreservation of duck semen. Int. J. Poult. Sci. 4, 197-201.

Harris, G.C., Benson, J.A. & Sellers, R.S., 1980. The influence of day length, body weight and age on the production ability of breeder cockerels. Poult. Sci. 63, 1705-1710.

Hiemstra, S.J., Van Der Lende, T. & Woelders, H., 2005. The potential of cryopreservation and reproductive technologies for animal genetic resources conservation strategies. The role of biotechnology. Villa Giuliana, Turin, Italy. FAO. Rome, Italy.

Holt, W.V., 2000. Basic aspects of frozen storage of semen. Anim. Reprod. Sci. 62, 3-22.

Islam, M.S., Howlider, M.A.R., Kabir, F. & Alam, J., 2002. Comparative assessment of fertility and hatchability of barred Plymouth rock, white leghorn, Rhode island red and White rock hens. Int. J. Poult. Sci. 1, 85-90.

Karaca, A. G., Parker, H.M. & McDaniel, C.D., 2002. Elevated body temperature directly contributes to heat stress infertility of broiler breeder males. Poult. Sci. 81, 1892-1897.

Kleven, O., Fossoy, F., Laskemoen, T., Robertson, R.J., Rudolfsen, G. & Lifield, J.T., 2009. Comparative evidence for the evolution of sperm swimming speed by sperm competition and female sperm storage duration in Passerine birds. Evol. 63, 2466-2473.

Kotlowska, M., Glogowski, J., Dietrich, G.J., Faruga, A., Jankowski, J. & Ciereszko, A., 2005. Biochemical characteristics and sperm production of turkey semen in relation to strain and age of the males. Poult. Sci. 84, 1763-1768.

Koyanagi, F. & Nishiyama, H., 1981. Disintegration of spermatozoa in the infundibular sperm-host glands of the fowl. Cell Tis. Res. 214, 81-87.

Lake, P.E., 1983. Factors affecting the fertility level in poultry, with special reference to artificial insemination. Agric. Research Council. U.K.

Latif, A., Ijaz, A., Aleem, M. & Mahmud, A., 2005. Effect of osmotic pressure and pH on the short-term storage and fertility of broiler breeder sperm. Pak. Vet. J. 25, 179-183.

Lisowski, M. & Bednarczyk, M., 2005. Effects of tamoxifen dose and nutrition scheme during growth on stimulation of the reproductive system in Cornish breed cocks. Folia Biol. (Krakow), 53, 1-6.

Long, J.A., 2006. Avian semen cryopreservation: what are the biological challenges? Agric. Research service.

Lukaszewicz, E., 2002. An effective method for freezing white Italian gander semen. Theriogenology, 58, 19-27.

Lukaszewicz, E. & Kruszynski, W., 2003. Evaluation of fresh semen and frozen-thawed semen of individual ganders by assessment of spermatozoa motility and morphology. Theriogenology, 59, 1627-1640.

Lukaszewicz, E., Jersey, A., Partyka, A. & Siudzinska, A., 2008. Efficacy of evaluation of rooster sperm morphology using different staining methods. Res. Vet. Sci. 85, 583-588.

Machebe, N.S. & Ezekwe, A.G., 2005. Ejaculate characteristics of three genotypes of local cocks in the humid tropics. J. Agric, Food, Env. Ext. 3, 33-37.

Makawi, S.A., Elsharif, B.A. & Babiker, E.A. 2007. Effect of season on freezability of semen from two breed-types of desert sheep in the Sudan. J. Anim. Vet. Adv. 6, 846-849.

Martin, R.D., 2004. Artificial insemination of poultry. Poultry Essays. Essay 14.

Maule, J.P., 1962. The semen of animals and artificial insemination. Common Wealth Agricultural Bureaux England.

Molekwa, J.T., 2007. Relationships between cock semen viability and the fertility of artificially inseminated South African indigenous chicken breeds. Dissertation M. Tech. Degree, CUT, Free State. Bloemfontein.

Molekwa, J.T. & Umesiobi, D.O., 2009. Relationships between cock semen viability and the fertility of artificially inseminated South African indigenous chicken breeds. S.A. J. Anim. Sci. 39, 24-28.

Mouldin, M.J., 2000. Fertilization of the avian embryo. Cooperative extension service.

NAFU, Farmer Technology, 2008. How to start a poultry farm. http://www.nafufarmer.co.za/.pdf-files. (Accessed on 27 Apr. 2009).

Nishiyama, H., 1961. On the quality of the cock semen obtained by different collection methods. Mem. Fac. Agric. Kagoshima Univ. 4, 43-50.

Nwachukwu, E., N., Ibes. N. & Amadi, C.U., 2006. Effects of genotype and frequency of semen collection on semen characteristics of local chicken cocks. J. Anim. Vet. Adv. 5, 562-565.

Obidi, J.A., Onyeanusi, B.I., Rekwot, P.I., Ayo, J.O. & Dzenda, T., 2008. Seasonal variations in seminal characteristics of Shikabrown breeder cocks. Int. J. Poult. Sci. 7, 1219-1223.

Oguntunji, A.O., Aderemi, F.A., Lawal, T.E. & Alabi, O.M., 2008. The influence of seasonal variation on performance of a commercial laying strain in a derived savanna environment in Nigeria. Nig. J. Poult. Sci. 5, 75-82.

Parker, J.E. & Arscott, G.H., 1963. Energy intake and fertility of male chickens. J. Nutr. 82, 64.

Parker, H.M. & McDaniel, C.D., 2002. Selection of young broiler breeders for semen quality improves hatchability in an industry field trial. Poult. Sci. 11, 250-259.

Parker, H.M., Karaca, A.G., Yeatman, J.B., Frank, L.R. & McDaniel, C.D., 2002. Fertility of Broiler Breeders Following Categorization by the Optibreed Sperm Quality Index When Hens Are Inseminated with a Constant Number of Sperm. Poult. Sci. 81, 239-245.

Parker, H.M. & McDaniel, C.D., 2006. The immediate impact of semen diluent and rate of dilution on the sperm quality index, ATP utilization, gas exchange and ionic balance of broiler breeder sperm. Poult. Sci. 85, 106-116.

Parker, T.H., Thompson, D., Ligon, J.D., Schneider, B. & Byrn, F., 2006. Does red jungle fowl comb size predict sperm swimming speed and motility? Eth. Ecol. Evol. 18, 53-60.

Perry, E.J., 1960. The Artificial Insemination of Farm Animals. Rutgers University Press, New Jersey.

Peters, S.O., Shoyebo, O.D., Ilori, B.M., Ozoje, M.O., Ikeobi, C.O.N. & Adebambo, O.A., 2008. Semen quality traits of seven strain of chickens raised in humid tropics. Int. J. Poult. Sci. 7, 949-953.

Petek, M. & Dikmen, S., 2006. The effects of prestorage incubation and length of storage of broiler breeder eggs on hatchability and subsequent growth performance of progeny. Czech J. Anim. Sci. 51, 73-77.

Purdy, P.H., 2006. A review on goat sperm cryopreservation. Small Rumin. Res. 63, 215-225.

Reis, L.H. & Soares, M.C., 1997. Effects of short storage conditions and broiler breeder age on hatchability, hatching time, and chick weights. Poult. Sci. 76, 1459-1466.

Renema, R.A., Rustad, M.E. & Robinson, F.E., 2007. Implications of changes to commercial broiler and broiler breeder body weight targets over the past 30 years. Wld. Poult. Sci. 63, 457-472.

Saacke, R.G., 1983. Semen quality in relation to semen preservation. Dept. Dairy Sci. 66, 2635-2644.

Sadanand, D., Sontakke, G.U., Vedantam, S., Sanjiva, D. & Kholkute, S.S., 2004. Semen characteristics, cryopreservation and successful artificial insemination in the Blue rock pigeon (Columba livia). Theriogenology, 62, 139-153.

Salisbury, G.W., Van Demark, N.L. & Lodge, J.R., 1978. Physiology of reproduction and artificial insemination of cattle. (2nd ed.) San Francisco. W.H. Freeman and Company. USA.

SAS, 2006. Statistical Analysis System user's guide. SAS Institute Inc., Raleigh, North Carolina. USA.

Seigneurin, F. & Blesbois, E., 1995. Effects of the freezing rate on viability and fertility of frozen-thawed fowl spermatozoa. Theriogenology, 43, 1351-1358.

Senger, P.L., 2003. Pathways to pregnancy and Parturition. (99164-6332) 2nd Ed. Pullman, Washington, USA

Sexton, T.J., 1977. A new poultry semen extender. Int. Poult. Sci. 56, 1443-1446.

Sexton, T.J., 1979. Possible commercial method of freezing chicken semen. J. Reprod. Fert. 55, 1-7.

Sexton, T.J., 1980. Optimal rate for cooling chicken semen from +5°C to -196°C. Poult. Sci. 59, 2765-2770.

Sexton, T.J., 1981. Sperm number required for maximum fertility of chicken semen processed for freezing. Reprod. Nutr. Develop. 21, 1043-1048.

Siudzinska, A. & Lukaszewicz, E., 2008a. The effect of breed on freezability of semen of fancy fowl. Anim. Sci. 26, 331-340.

Siudzinska, A. & Lukaszewicz, E., 2008b. Effect of semen extenders and storage time on sperm morphology of four chicken breeds. Appl. Poult. Res. 17, 101-108.

Surai, P.F. & Wishart, G.J., 1996. Poultry artificial insemination technology in the countries of the former USSR. Wld. Poult. Sci. J. 52, 29-43.

Tabatabaei, S., Batavani, R.A. & Talebi, A.R., 2009. Comparism of oviductal sperm age on fertility, hatchability and embryonic death rates in Iranian indigenous and ross-308 broiler breeder chickens. J. Anim. Vet. Adv. 8, 85-89.

Tai, J.J.L., Chen, J.C., Wu, K.C., Wang, S.D. & Tai, C., 2001. Cryopreservation of gander semen. Br. Poult. Sci. 42, 384-388.

Taylor, L.W., 2003. Fertility and hatchability of chicken and turkey eggs. Lucknow. Greenworld publishers, India.

Tselutin, K., Seigneurin, F. & Blesbois, E., 1999. Comparison of cryoprotectants and methods of cryopreservation of fowl spermatozoa. Poult. Sci. 78, 586-590.

Tuncer, P.B., Kinet, H., Ozdogan, N. & Demiral, O., 2006. Evaluation of some spermatological characteristics in Denizli cocks. J. Fac. Vet. Med. Univ. 3, 37-42.

Tuncer, P.B., Kinet, H. & Ozdogan, N., 2008. Evaluation of some spermatological characteristics in Gerze cocks. Ankara Univ. Vet. Fak. Derg. 55, 99-102.

USDA (United States Department of Agriculture), 2007. South Africa Poultry and Product Annual 2007. USDA Foreign Agricultural Service. http://www .thepoultrysite. com /articles /922/south-africa-poultry-andproducts-annual -2007. Accessed on 24 November 2007.

Verstegen, J., Iguer-ouada, M. & Onclin, K., 2002. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology, 57, 149-179.

Vyt, P., 2007. Examination and storage of liquid porcine semen. Faculty of Vet. Med. Ghent University. PhD. Thesis.

Watson, P.F., 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 60-61, 481-492.

Wilson, H.R., 1997. Effects of maternal nutrition on hatchability. Poult. Sci. 76, 134-143.

Wilson, H.R., Piesco, N.P., Miller, E.R. & Nesbeth, G.W., 1979. Prediction of the fertility potential of broiler breeder males. Wld. Poult. Sci. J. 35, 95-118.

Woelders, H., Zuidberg, C.A. & Hiemstra, S.J., 2006. Animal genetic resources conservation in the Netherlands and Europe: poultry perspective. Poult. Sci. 85, 216-222.

Yang, L., Mutanen, M., Cheng, Y. & Duan, R., 2002. Effects of red meat and fiber in high fat diet on activities of sphingomyelinase, ceramidase and caspase-3 in rat colonic mucosa. Nutr. Biochem. 13, 499-504.

Yeste, M., Briz, M., Pinart, E., Sancho, S., Garcia-Gil, N., Badia, E., Bassols, J., Pruneda, A., Bussalleu, E., Casas, I. & Bonet, S., 2008. Boar spermatozoa and prostaglandin $F_{2\alpha}$ Quality of boar sperm after the addition of prostaglandin $F_{2\alpha}$ to the short-term extender over cooling time. Anim. Reprod. Sci. 108, 180-195.

Zahraddeen, D., Butswat, I.S.R., Kalla, D.J.U., Sir, S.M. & Bukar, M.T., 2005. Effect of frequency of ejaculation on semen characteristics in two breeds of turkeys (*Meleagris gallopavo*) raised in a tropical environment. Int. J. Poult. Sci. 4, 217-221.