# DEVELOPMENT OF CRYOPRESERVATION STRATEGIES FOR IMPROVED REPRODUCTIVE COMPETENCE IN SOUTH AFRICAN PIG GENOTYPES

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# DEVELOPMENT OF CRYOPRESERVATION STRATEGIES FOR IMPROVED REPRODUCTIVE COMPETENCE IN SOUTH AFRICAN PIG GENOTYPES

by

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I, Matshidiso Bailekae Masenya, ID: 8608240913089, student number: 209082097, declare that this dissertation: **Development of cryopreservation strategies for improved reproductive competence in South African pig genotypes** submitted to the Central University of Technology, Free State for the degree MAGISTER TECHNOLOGIAE: AGRICULTURE is my own independent work and that all sources used and quoted have been duly acknowledged by means of complete references; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology; and has not been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification. I also disclaim this dissertation in the favour of the Central University of Technology, Free State.

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Matshidiso B Masenya DATE

## DEDICATION

In dedication to my family, friends and staff of Germplasm Conservation & Reproductive Biotechnologies whom all contributed in so many ways to make this an incredible experience, the gain of knowledge, and tremendous personal growth.

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## LIST OF ACRONYMS AND ABBREVIATIONS

| ABBREVIATIONS | DESCRIPTION |
|---|---|
| AI | Artificial Insemination |
| ART | Assisted Reproduction Technology |
| BSA | Bovine Albumin Serum |
| BTS | Beltsville Thawing Solution |
| CASA | Computer Assisted Semen Analysis |
| EDTA | Ethylenediaminetetraacetic Acid |
| KCl | Potassium Chloride |
| LIN | Linearity |
| MEDIUM | Percentage of sperm with medium velocity |
| MOTILE | Percentage of motile sperm |
| $NaHCO_3$ | Sodium Bicarbonate |
| NPM | Non progressive motility |
| PM | Progressive Motility |
| RAPID | Percentage of rapidly moving sperm |
| SD | Standard deviation |
| SLOW | Percentage of slow moving spermatozoa |
| STATIC | Percentage of static sperm |
| STR | Straightness |
| VAP | Velocity Average Pathway |
| VSL | Velocity Straight Line |
| VCL | Velocity Curvilinear |

## GENERAL ABSTRACT OF THE STUDY

Frozen-thawed boar sperm has the potential to impact the future of the swine industry. The objectives of the study were to characterise semen of Kolbroek and Large White boars, find a suitable holding temperature and extender, determine the effect of breed and pregnancy rate following artificial insemination by frozen-thawed boar semen. A total of eight boars and 33 sows were used in this study. The 120 ejaculates were collected from each individual Kolbroek and Large White boars with the gloved-hand technique. Macroscopic and microscopic sperm characteristics were recorded. The bodyweight of Kolbroek (154.7 kg) was significantly lower compared to Large White (189.9 kg) boar. However, no significant differences were observed in Kolbroek and Large White boar semen volume (140 and 170 ml), sperm concentration (0.727 and 0.761 x $10^9$ sperm/ml), and total sperm motility (95 and 91%). A positive correlation existed between bodyweight and semen volume of Kolbroek (r= 0.22) and Large White (r= 0.26). Conversely, the bodyweight of Large White was positively correlated to sperm concentration of Large White (r= 0.37) but negatively correlated to Kolbroek (r= -0.66). Storage time and temperature did not affect Large White boar sperm motility rate. However, Kolbroek sperm total motility rate (61.0%) was affected at 25°C after 24 hours. The highest total sperm motility rate was observed for semen diluted with Tris-based extender (74.1%) in Kolbroek boars at 48 hours of storage. Large White boar semen diluted with BTS (62.9%), Kobidil$^+$ (69.3%) and Tris (65.1%) showed significantly higher sperm motility rate at 48 hours of storage, compared to Citrate (27.6%) extender. Cryopreservation significantly reduced sperm motility rate for Kolbroek (30.2%) and Large White (24.0%) boars. However, a high pregnancy rate was recorded in both sows of inseminated with raw diluted (100% vs. 81.3%) and frozen-thawed (50% vs. 50%) semen of Kolbroek and Large White boars. In conclusion, the bodyweight of Kolbroek and Large White boar was positively correlated with ejaculated semen volume. Kolbroek and Large White boar sperm stored at 18°C for 24 hours maintained the acceptable sperm motility rate. Kolbroek boar semen diluted with Tris-based extender maintained high sperm motility rate. Cryopreservation significantly reduced sperm motility rate regardless of breed; however, pregnancy rate from frozen-thawed semen was high for Kolbroek (50%) and Large White (50%) even though the frozen-thawed fertility was low.

**Keywords:** semen, Kolbroek, Large White, extenders, cryopreservation

# CHAPTER 1

# CHAPTER 1: BACKGROUND AND OVERVIEW OF THE STUDY

## 1. GENERAL INTRODUCTION

South Africa has a unique pool of indigenous livestock. According to Nedambale *et al.* (2008) the erosion of the gene pool of indigenous livestock breeds in South Africa is a cause of concern as they represent a valuable contribution to the rich biodiversity of the land. In Southern Africa, the diversity of the pigs, the breeds and populations, the farming systems and production environments that gave rise to the breeds, the characteristics peculiar to the breeds and their likely future role, are largely undocumented (Halimani *et al*., 2010).

Large White pigs are the most popular exotic breed in South Africa (Agricultural Research Council, 1993) and are the most common imported pigs in commercial pig production industry, due to their superior fertility and growth rates (Ncube *et al*., 2003). However, their high nutrient requirements (Umesiobi, 2008, 2009) and the need for intensive management systems (Thomas *et al*., 2010), make them unsuitable for resource-poor rural farmers and the future challenges of global warming. Kolbroek is a South African's indigenous pig breed, but very little research work has been done to characterize it (Farm Animal Conservation Trust, 2006). Indigenous pigs possess unique genetic traits such as diseases tolerance, longevity and adaptability in harsh environmental conditions (Ramsay *et al*., 1994).

There is general concern that the genetic variation within Kolbroek pig breed is becoming extinct. Reasons for the high extinction rate are manifold and interrelated. They include the large-scale promotion of uniform high-yielding breeds and crossbreeding; crossbreeding policies and natural disasters (Geerlings *et al*., 2002). The farmers also tend to keep the herd sizes small in order to adequately meet the animals' nutrition need (Thomas *et al*., 2010), leading to small populations that are vulnerable to inbreeding and natural disasters (Halimani *et al*., 2010). Most of the research has been focusing on the imported genotypes which cannot be sustained under smallholder conditions (Ncube *et al*., 2003). Research to evaluate the indigenous pig has been erratic and inadequate. Hence there is a need to evaluate

the reproductive potential of the South African indigenous boars in comparison with imported boars.

The reproductive potential of the indigenous Kolbroek boars has not been fully exploited in South Africa compared to other pig genotypes. Artificial insemination (AI) is one of the developments that have made a major contribution to both animal improvement and the conservation of farm animal genetic resources. However, successful application of AI technique requires identification of a suitable semen extender and temperature, a suitable glycerol concentration and the development of freezing protocols for semen cryopreservation. The effect of storage time of boar semen in liquid has been investigated in several studies (Waberski *et al*., 1994; Johnson *et al*., 2000). The storage tolerance of sperm, without noticeable decrease in quality, depends, among other factors, on the choice of extender (Levis, 2000).

Storage of semen and choice of extender are crucial for semen distribution and to optimize efficient use of semen for AI. In vivo, sperm cells are diluted with seminal fluids from the accessory glands at ejaculation and their motility is retained for a few hours (Johnson *et al*., 2000). To extend their survival *in vitro*, it is necessary to reduce the metabolic activity by chemical inhibitors or by lowering the temperature, which also requires dilution (Johnson *et al*., 2000). The maximum recommended storage time of semen is associated with the dilution level and the number of spermatozoa in the AI dose (Johnson *et al*., 2000).The acceptance of AI technology worldwide provided the impetus for developing other technologies, such as sperm cryopreservation.

Cryopreservation is defined as the freezing of tissues or cells to preserve them indefinitely for future use (Dinnyes *et al*., 2007). Cryopreserved boar semen has been available since 1975, a major breakthrough in commercial application has not yet occurred (Groβfeld *et al*., 2008). Boar semen cryopreservation could play an important role in pig breeding and genetic resources conservation. However, each protocol uses its own set of cryobiological problems in terms of sperm cell survival (Hofmo & Almlid, 1991) and therefore, the need of cryopreserved boar sperm is increasing; this decrease in sperm function becomes a more evident problem.

To date, the use of frozen-thawed semen for AI is considered a basic component for cattle breeding worldwide (Curry, 2000); however, the situation in the pig industry is completely different as the use of frozen-thawed boar semen is limited to less than 1% of the AI performed around the world (Wagner & Thibier, 2000). Previous reports suggested that post thawed sperm motility was ≥ 50% (Roca *et al*., 2006). Among these reasons behind this restricted use of frozen-thawed boar semen compared to liquid preserved ones, the frozen-thawed sperm have a shorter lifespan *in vitro* (Johnson *et al.*, 2000). Hence, it is imperative to develop a cryopreservation strategy in order to improve frozen-thawed survival rate.

## 1.2 MOTIVATION OF THE STUDY

The reproductive potential of the indigenous boars have not been exploited in South Africa, as much as it has been done in other pig breed genotypes. Moreover, lack of accurate method for sperm analysis to determine reproductive potential of indigenous boars may be not yet late as they are facing extinction. Thus it is critical to conserve their genetic materials as they possess unique gene pool with traits of economic importance. Indigenous pigs are adapted to local environment and poor quality feed but their numbers are declining largely due to livestock production policies that prefer the use of fast-growing imported breeds. It has been established that indigenous pigs are adapted to poor quality nutrition as compared to European breeds (Chimonyo & Dzama, 2007). Therefore it is of utmost importance to cryopreserve their sperm for future use in breeding program.

## 1.3 PROBLEM STATEMENT

There is little or no information on cryopreservation of South African indigenous pigs. Cryopreservation of boar semen remains low and a challenging obstacle.

## 1.4 PRIMARY OBJECTIVE

Development of cryopreservation strategies for boar semen and subsequently improve reproductive performance following artificial insemination.

## 1.5 SPECIFIC OBJECTIVES

- Comparisons of South African Indigenous and Exotic Boar Breeds on Sperm Parameters following Computer Aided Sperm Analysis (CASA).
- Effect of *in vitro* storage temperature and period on Kolbroek and Large White boar sperm motility.
- Effect of extender and storage period on the South African indigenous Kolbroek and Large White boar sperm motility rate following analysis by Computer Aided Sperm Analysis.
- Preliminary comparative evaluation of the different glycerol concentrations on boar semen parameters prior to cryopreservation.
- High fertility rate following artificial insemination by frozen-thawed boar semen.

## 1.6 HYPOTHESIS

Development of cryopreservation strategies will improve boar semen viability and fertility rate following artificial insemination.

## 1.7 REFERENCES


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# CHAPTER 2

# CHAPTER 2: LITERATURE REVIEW

## 2.1 HISTORY AND ORIGIN OF BREEDS

### 2.1.1 KOLBROEK PIGS

The Kolbroek pig breed resembles a breed of pigs that are commonly found in China. There is evidence that a sailing ship, belonging to the Dutch East India Company was wrecked off the coast of South Africa at Cape Hangklip and the pigs on board of sailing-ship fell into the hands of farmers who had settled in the area (Ramsay *et al*., 1994). The name of the ship was the Colebrook, and then the pig was named Kolbroek. Kolbroek pig is short in height with pricked ears and a squashed face. The breed is dark coloured being either black or brown and are often striped at birth (Figure 2.1).

**Figure 2.1** Kolbroek boar

The Kolbroek is an early maturing breed that grows slower than modern pig breeds and it has a higher back fat thickness at 222 days of age (South African Indigenous Breeds, 2009). They are ideally suited for free range in the smallholder systems. The Kolbroek pigs have good mothering ability and fertility rate, efficient converter of high roughage rations, have the ability to forage and digest root and leaf crops, the surplus fat can be reduced for sale as lard and crackling. They have a docile temperament with high stress tolerance (South African Indigenous Breeds, 2009). The average live body weight of a matured Kolbroek pig is 112 kg and an average litter size is seven to eight piglets (South African Indigenous Breeds, 2009).

**Table 2.1** Adding value to indigenous pigs – economically useful characteristics

| Characteristics | Value |
|---|---|
| Conversion of coarse fiber rations and root crops | Suitable for free range systems; less dependence on expensive high grain rations |
| Parasite tolerance | Less expense on stock remedies |
| Strong feet | Can be used to improve the feet of modern breeds with foot problems |
| Excess fat | Fat can be trimmed off the carcass and reduced for sale as lard and crackling |
| Meat | Niche market potential - tasty and additive free pork |

Source: Ramsay *et al*. (1994)

### 2.1.2 LARGE WHITE PIGS

Large White is the most popular breed in South Africa (Agricultural Research Council, 1993). It is an exotic breed and originates from the United States of America. It has white hair and a pink skin. It is well known for its excellent mothering and rearing ability of the sow as well as its docility. Large White pigs farrow an average of 10 piglets per litter (Umesiobi, 2010) and possess high percentage of piglet survival at 21 days of age (Browne, 1994). Compared to other breeds in South Africa, the Large White has high performance figures and is less susceptible to stress (Agricultural Research Council, 1993). Large White pigs are the most common imported pigs in commercial pig production, due to their superior fertility and growth rates (Ncube *et al*., 2003).

**Figure 2.2** Large White boar

## 2.2 CURRENT STATUS OF SOUTH AFRICAN INDIGENOUS LIVESTOCK

According to Nedambale *et al*. (2008), the indigenous livestock represent a valuable contribution to the rich biodiversity of the land and plays a major role in the social, cultural and economic history of the country. Maintaining a wide range of indigenous animal breeds is crucial to food security, poverty alleviation and sustainable development (Geerlings *et al*., 2002). These indigenous livestock often possess valuable traits such as disease tolerance, longevity and adaptability to harsh conditions and cheap feed sources (Umesiobi, 2000a, b) and kitchen wastes, such as maize cobs, vegetables (Umesiobi, 2000b; Kanengoni *et al*., 2002; Ndindana *et al*., 2002), all of which are qualities that form the basis for low-input, sustainable agriculture

Indigenous livestock breeds are currently subjected to fast degradation and dilution because of unplanned breeding, crossbreeding and introduction of exotic germplasm (Scholtz, 2005). They are being replaced by crossbred livestock with imported high-yielding breeds to increase performances, leading to a severe decrease in the number of indigenous breeds (Lemke *et al*., 2005). Their extinction would mean a considerable loss for indigenous biodiversity; genes of potential use for future breeding programs would be eliminated; and the food security and economy of smallholders might be put at risk.

### 2.2.1 FARM ANIMAL GENETIC RESOURCES

There are two broad approaches through which farm animal genetic resources (FAnGR) can be conserved: *ex situ* and *in situ*. *'Ex situ'* refers to conservation approaches outside of a breed's natural habitat – for example, in zoos and in gene banks (Geerlings *et al*., 2002). *'In situ'* is 'the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species in their natural surroundings (Geerlings *et al*., 2002).

Effective semen cryopreservation would ensure long-term availability of genetic resources, especially for those endangered species like the Kolbroek pig to assist biosecurity measures, encourage international gamete exchange, facilitate gender selection technology and permit rational gene banking (Geerlings *et al*., 2002). Hence, developing commercially acceptable protocols of semen cryopreservation would be in the best interests of the global swine industry. Livestock gene banking constitutes a reservoir of genetic adaptability that acts as a buffer against environmental changes, disease concerns, new knowledge of human nutrition needs, shifting market conditions and societal demands, all of which are largely unpredictable (Bailey *et al*., 2008). To establish such conservation measures, the development of genetic resource banks is essential to store frozen sperm, eggs and embryos from threatened populations, with the deliberate intention to use them in a breeding program.

## 2.3 COLLECTION OF BOAR SEMEN

Not all boars are capable of mounting a dummy, or will remain mounted to be collected at all or in a reasonable period of time. Boar semen can be collected by using a female that is in standing oestrus. This requires a female that is close to size matched for the boar to be collected. This can sometimes be inconvenient since boars can be overly aggressive and the oestrus female may refuse to stand for certain boars or inexperienced males that can frustrate females. Alternatively, a more convenient method involves collecting the boar by training him to be collected from a dummy. The boar learns to associate the dummy with ejaculation and pleasure after a training period and will mount the dummy when

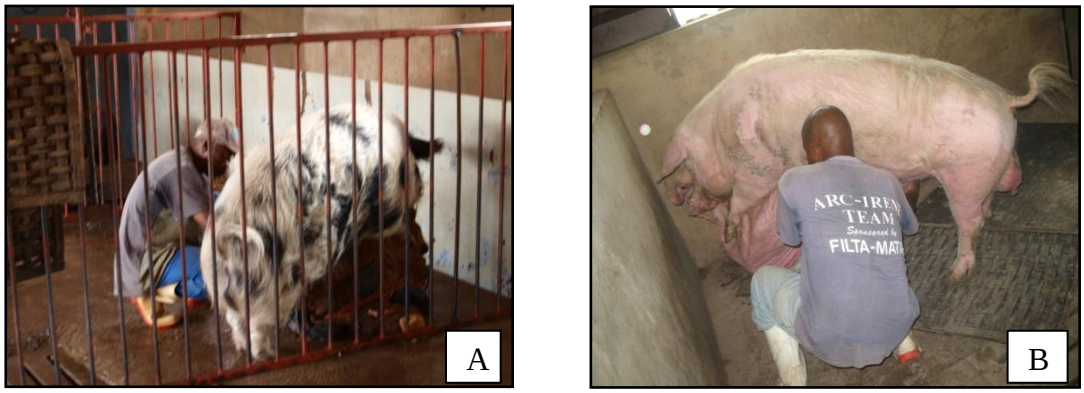


**Figure 2.3** Semen collections from Kolbroek (A) and Large White boars (B) using gloved hand technique.

## 2.4 SEMEN EVALUATION

Semen evaluation is an important tool in estimating the fertility of boars (Ogbuewu *et al*., 2007).

### 2.4.1 SEMEN VOLUME

Visual evaluation of the opacity of the ejaculate gives an idea on the sperm concentration (Shipley, 1999). Boar ejaculates vary between 150 and 300 mL (Garner & Hafez, 1986). The volume is subject to considerable variations as a result of individual characteristics and environmental conditions. In comparative terms, boar semen volume is higher than for other domestic species. In comparison, buck semen volume ranges between 0.2 and 2.5 ml (Setchell, 1991), and from 0.75 to 2 ml (Gil *et al*., 2003) for rams

Ejaculation of boars occurs in three phases namely; (i) pre sperm fraction consisting of 5-15 ml of a colourless water fluid containing few sperm, (ii) a sperm rich fraction which is milky white making up about 10-20% of the ejaculate volume containing up to 90% of the sperm could withstand handling procedures (extension, handling, and freezing-thawing) better than those contained in the latter part of a fractionated ejaculate (Zhu *et al*., 2000) (iii) post sperm fraction containing about 60-80% of the ejaculate volume (Tavener & Dunkin, 1996). The reasons for these differences,

although not yet disclosed in detail, may be related to differences in electrolyte composition or protein components (Zhu *et al*., 2000).

### 2.4.2 SEMEN pH

Johnson *et al*. (2000) reported that the pH of raw boar semen varies between 7.0 and 7.5. Major changes in boar semen pH can result in sperm damage, infertility, or sperm mortality (Purdy, 2006). The most buffering agent found in the seminal plasma is Bicarbonate that is produced by the vesicular gland, but this is meant to occur in vivo and not under in vitro conditions which occur during cryopreservation (Purdy, 2006). For this reason, substances that buffer pH changes are routinely included in cryopreservation media to minimize such changes.

According to Jones & Connor (2004), the glycolytic pathway in boar sperm is sensitive to pH, which decreases as lactate is produced from either glucose or fructose in vitro hence the build-up of lactate appears to be due to the saturation of mitochondrial lactate transporters, which causes the cytoplasmic pH to fall. Infection is usually associated with alkaline ejaculate (pH >8.0). Bacterial contamination mainly leads to a series of alterations including diminished sperm motility, an increased proportion of altered acrosomes and pH lowering to acidic levels (5.7-6.4) (Althouse *et al*., 2000). The addition of buffering agents therefore helps control the pH of the medium.

Generally, there are four basic parameters that are measured to evaluate boar semen quality and they are: (1) Sperm concentration, (2) Percentage sperm motility, (3) Sperm morphology and (4) Acrosomal integrity.

### 2.4.3 SEMEN CONCENTRATION

The number of sperm in a semen dose is important for the fertilization process. Measuring semen concentration or total numbers of sperm is not an element of semen quality evaluation, but more so, as a tool to monitor the health and productive output of the boar (Ogbuewu *et al*., 2007) and as the primary feature in processing boar ejaculates for optimizing the genetic potential of a single individual. However,

variation in the number of sperm in an ejaculate has been described between different pig breeds (Kommisrud *et al*., 2002), which is a first factor influencing semen dose production. Not only differences in sperm number but also in sperm volume, ranging from 150 to 300 ml (Kondracki, 2003), influence sperm concentration. Individual variation within a breed is also very important (Johnson *et al*., 2000).

Physiological limitations include the inability to store boar semen for long periods without significant reductions in its fertilizing capacity (Johnson *et al*., 2000) and the need to use large numbers of sperm in insemination doses compared with other livestock species (Flowers, 1998). The number of sperm inseminated determines the boar's impact on litter size (Umesiobi, 2008). Increasing litter size on operations using natural service is most likely to result from improvements in the quality of sperm. This is due to the fact that most boars contain sufficient sperm in their ejaculate to maximize sperm numbers in the oviduct (Flowers, 2002; Ogbuewu *et al*., 2007).

### 2.4.4 SPERM MOTILITY

Motility is known to be an important characteristic in predicting the fertilizing potential of an ejaculate (Holt *et al*, 1997; Gadea, 2005). Changes in sperm movement patterns can reflect physiological events within the sperm (Cremades, 2005; Umesiobi, 2010). The simplest way to evaluate sperm motility is by estimating the number of motile sperm under a light microscope or using phase contrast microscopy (Shipley, 1999). This method is very subjective since it depends on the interpretation of the motility by an individual (Woelders, 1991). It is inexpensive and quick, but accuracy depends on the subjective estimation by individuals even though surprisingly consistent results can be obtained (Woelders, 1991). Objective Computer Aided Sperm Analysis® (CASA®) systems have become commercially available, but these systems are not frequently used in commercial AI-centers because of the high investment costs (Verstegen *et al*., 2002).

As boar sperm show a higher percentage of circular movement than those from other species, except stallions, it is recommended to estimate the different forms of

motility, including proportions of progressive sperm (Johnson *et al*. 2000). When sperm are released into seminal plasma in vivo or into physiological medium in vitro, they rapidly begin to swim vigorously in a nearly straight trajectory. Hyper activated sperm tend to swim vigorously in circles on a microscope slide (Ho & Suarez, 2001).

### 2.4.5 SPERM MORPHOLOGY

The microscopic appearance of sperm can give information on morphological abnormalities, cell membrane integrity and the acrosome (Woelders, 1991). Sperm morphology is of great concern because infertility is often due to a high proportion of structurally abnormal sperm (Barth, 1995; Umesiobi, 2010).

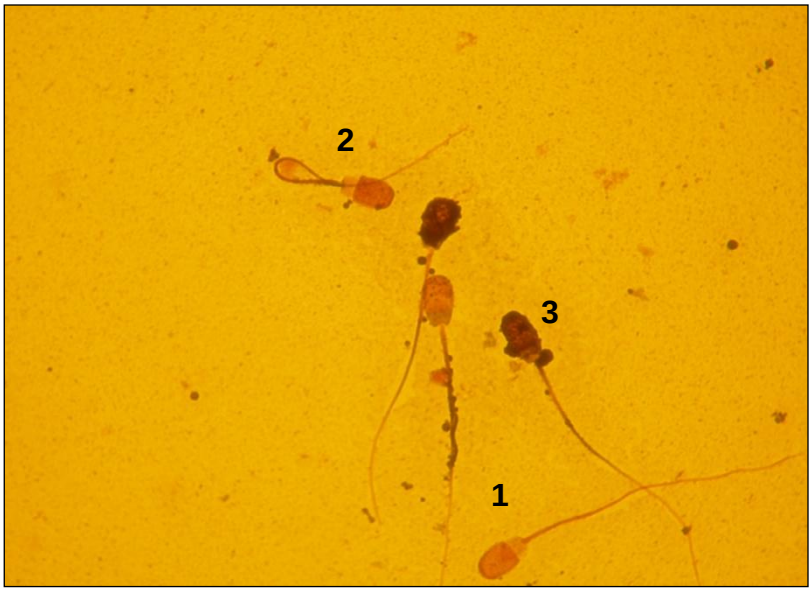


**Figure 2.4** Kolbroek sperm viability and morphology using Giemsa staining solution: (**1**) Live sperm, (**2**) Live and abnormal sperm-coiled tail, (**3**) Dead sperm

Primary morphological abnormalities, or those that affect the sperm head region, as a result of a disturbance in spermatogenesis, contribute a significant role in determining the population of sperm that reach the site of fertilization. Remainders of cytoplasm, proximal or distal droplets, and small tail abnormalities are defined as secondary abnormalities and can be compensated for by the semen dose (Donadeu, 2004). Criteria for the maximum percentage of primary and secondary abnormalities in commercial porcine AI-centres were determined as 10% and 20%, respectively

(Waberski *et al*., 1994a; Flowers, 1997; Shipley, 1999). As demonstrated in Figure 2.4, the percentage of sperm with normal morphology should be at least 70% (Shipley, 1999). The detrimental effect of high percentages of morphologically abnormal sperm on overall embryo development could in part be related to defective binding of sperm to the zona pellucida that is essential for fertilization, and this process being highly selective against sperm with abnormal morphology (Amann *et al*., 1999). Thundathil *et al*. (1999) reported that abnormal sperm that penetrates the oocyte can be involved in the fertilization process, but the resulting zygotes may be less competent.

**Table 2.2** Overview of the criteria for use of pig semen in artificial insemination for optimum sows fertility.

| Semen parameters | Range of sperm quality requirements | | | |
|---|---|---|---|---|
| | **Kuster & Althouse, 1999** | **Martin-Rillo *et al*, 1996** | **Shipley, 1999** | **Britt *et al*, 1999** |
| Motility | >70 | 60-100 | >70 | >60 |
| Abnormal morphology | <20 | | <20 | |
| Normal acrosome | | 70-100 | | |
| Cytoplasms droplets | <15 | | <15 | |
| Proximal droplets | | 0-20 | | |
| Coiled tails | | 0-30 | | |
| Primary abnormalities | | 0-4 | | <10 |
| Secondary abnormalities | | | | <20 |

* Recommendation for 2 x $10^9$ sperm per dose.

## 2.5 HOLDING TIME

The most commonly used method of semen storage in the pig industry is the liquid-storage. One of the main deterrents to the wide-spread use of AI in swine is short storage of boar sperm. Storage of semen doses for a certain period is necessary for their distribution and to optimize efficient use of semen for AI. It is of practical and economic importance that the semen storage time does not negatively influence fertility. However, the natural ageing process cannot be prevented in liquid diluted boar semen, not even during the first days of storage (Waberski *et al*., 1994a; Johnson *et al*., 2000). A suppression of sperm metabolic activity, to reduce energy consumption and by-product formation, is needed to ensure sperm longevity (Althouse *et al*., 1998). Maintaining extended semen at a temperature between 15 °C and 20 $^{o}$C has been reported as optimal for storage of liquid boar semen (Paulenz *et al*., 2000).

Liquid-storage generally preserves semen at an acceptable quality for up to 7 days, depending on quality of semen and type of extender used. The advantage of this method is that it is proven to give farrowing rates highly comparable with natural mating; the results being better over shorter than longer storage periods (Weitze, 2000). Freshly ejaculated boar sperm in the whole semen ejaculate will not survive even slow cooling below 15ºC, with an increase in the number of cells affected and the magnitude of the damage as the temperature approaches 0ºC.

## 2.6 DILUENTS

According to Purdy (2006), the purpose of a cryopreservation diluent is to supply the sperm with sources of energy, protect the cells from temperature-related damage, and maintain a suitable environment for the sperm to survive temporarily. At a practical level and for current production purposes, diluents can be divided into two major groups: those designed for short-term preservation (less than one to three days), and diluents for long-term semen preservation (over four days) (Table 2). Depending on the composition of the extender, semen can be stored for two to three days in short-term extenders and up to five days or longer in long-term extenders (Johnson *et al*., 2000). Long-term extenders differ from short-term extenders mainly

by the use of complex buffering systems (HEPES, Tris), mostly in addition to the bicarbonate buffering system, and by the presence of Bovine Serum Albumin (BSA). The latter has a positive influence on sperm survival (Johnson *et al*., 2000) due to the absorption of metabolic bacterial products from the extender.

The intracellular pH of sperm is lowered which reduces their motility and enables them to survive several days at ambient temperature (Johnson *et al*, 2000). Glucose also contributes to large extent to the osmotic equilibrium (Johnson *et al*., 2000). Ethylenediamine-tetra-acetic acid (EDTA), a chelating substance, captures divalent metal ions, especially $Ca^{++}$, and is believed to limit their movement across the plasma membrane (Watson, 1990), preventing the initiation of capacitation and the acrosome reaction. The ions in media for fresh semen are introduced as sodium bicarbonate and sodium citrate and, in certain diluents, potassium chloride.

**Table 2.3** Composition (L) of different extenders for short-term (BTS, Kiev) and long-term (Androhep, Modena, Zorlesco) use in fresh porcine semen

| **Composition** | **Short-term extender** | | **Long-term extender** | |
|---|---|---|---|---|
| | **Beltsville Thawing Solution (BTS) (Pursel & Johnson, 1975)** | **Androhep (Weitze, 1990)** | **Modified Modena (Johnson et al., 1988)** | **Zorlesco (Gottardi, et al., 1980)** |
| Glucose | 37.00 g | 26.00 g | 25.00 g | 11.50 g |
| Fructose | | | | |
| Sodium citrate | 6.00 g | 8.00 g | 6.90 g | 11.70 g |
| $NaHCO_3$ | 1.25 g | 1.20 g | 1.00 g | 1.25 g |
| EDTA | 1.25 g | 2.40 g | 2.25 g | 2.30 g |
| KCl | 0.75 g | | | |
| Hepes | | 9.00 g | | |
| BSA | | 2.50 g | 3.00 g | 5.00 g |
| Gentamycin | | | | |
| Citric acid | | | 2.00 g | 4.10 g |
| Tris buffer | | | 5.65 g | 6.50 g |
| Cysteine | | | 0.5 g | 0.10 g |

$NaHCO_3$ (Sodium bicarbonate); EDTA (Ethylenediaminetetraacetic acid); KCl (Potassium Chloride); BSA (Bovine serum albumin).

## 2.7 CRYOPROTECTANTS

Cryoprotectants are reagents used to protect biological cells or tissues against cryopreservation damage (Li *et al*., 2005). They are included in cryopreservation medium to reduce the physical and chemical stresses derived from cooling, freezing and thawing of sperm (Purdy, 2006). Since the discovery of the cryoprotective properties of glycerol (Bailey *et al*., 2000), its choice as a penetrating cryoprotectant has been confirmed as the most effective in lowering the intracellular water freezing point. The cryoprotective benefits of glycerol on sperm are attributed mostly to its water-binding properties (Salomon & Maxwell, 1995). Since then, the use of glycerol to preserve sperm during freezing is widespread (Hammerstedt *et al*., 1990; Bailey *et al*., 2000).The effects of cooling velocity and cryoprotectant concentration on post thaw fertility have been investigated in several species (Katkov *et al*., 1999).

Cryoprotectants are classified as either penetrating or no penetrating. Penetrating cryoprotectants (glycerol, dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol) cause membrane lipid and protein rearrangement, resulting in increased membrane fluidity, greater dehydration at lower temperatures, reduced intracellular ice formation, and increased survival to cryopreservation (Holt, 2000). Additionally, penetrating cryoprotectants are solvents that dissolve sugars and salts in the cryopreservation medium (Purdy, 2006).

Glycerol is a permeating agent that enters into the cell to replace the water that leaves during cooling, thus reducing the osmotic stress (Medeiros *et al*., 2002). However, permeating cryoprotective agents, such as glycerol, can be toxic to the cell and cause membrane instability and motility loss (Medeiros *et al*., 2002). The toxic effects are due to high glycerol concentrations or too rapid of an addition and removal from the cell (Katkov *et al*., 1999). Due to this toxic effect of glycerol, it is important to make certain the optimal concentration to give a cryoprotective effect while minimizing damage to the cell. At each glycerol level, sperm can tolerate a variety of temperature ranges without viability losses (Johnson *et al*., 2000).

A non-penetrating cryoprotectant (egg yolk, nonfat skimmed milk, trehalose, aminoacids, dextrans, sucrose) doesn’t cross plasma membrane and only acts

extracellularly (Aisen *et al*., 2000). Therefore, non-penetrating cryoprotectant may alter the plasma membrane, or act as a solute, lowering the freezing temperature of the medium and decreasing the extracellular ice formation (Amann *et al*., 1999). Egg yolk phospholipids can lessen chilling injury on sperm by binding to low density lipoproteins of the membrane and by increasing the permeability of the membrane, although they do not alter intrinsic membrane composition and/or physical properties (Holt, 2000).

## 2.8 CRYOPRESERVATION OF BOAR SEMEN

Preserving genetic diversity in swine is important for the maintenance of healthy production populations, as well as the conservation of rare breeds that have genetic traits of merit. Long-term storage of semen brings forth additional advantages to producers of agriculturally important animals. Of particular importance to the pig industry, the use of frozen semen would help to control transmission of certain pathogens (Bailey *et al*., 2008), facilitates the distribution of agriculturally desirable genes, transportation of semen over long distances and continued use of the sire that is dead or ill (Bailey *et al*., 2008). Successful cryopreservation of boar semen is also necessary for international sales. According to Article 3.2.2.6 of the Terrestrial Animal Health Code (World Animal Health Organization, 2006) the conditions applicable to the packing and storage of porcine semen state that semen for export should be stored separately in liquid nitrogen in sterilized flasks for at least 28 days.

Despite these potential advantages of long-term semen storage, porcine sperm are notoriously sensitive to cold shock, and frozen-thawed semen is not routinely used by the industry (Bailey *et al*., 2008). Farrowing rates decrease by 20–30% and litter size drops by two to three piglets when using cryopreserved semen, in comparison to rates attained using liquid semen because approximately 40–50% of the sperm do not survive cryopreservation (Watson, 2000). Even using similar numbers of motile sperm, fertility is still poorer than with raw semen, due to sub lethal damage (Watson, 2000). Many of the frozen-thawed sperm show a shorter life span and have difficulties in reaching the oocytes and penetrating their vestments after conventional AI (Cremades *et al*., 2005). Such impairment could be due to changes in motility patterns following semen handling during cryopreservation (Cremades *et al*., 2005).

## 2.9 CRYO-INJURIES

During the process of cooling, freezing and thawing, sperm are subjected to a series of drastic changes in their physical and chemical environment (Watson, 2000). It involves subjecting the sperm to a series of closely related steps, mainly extension and concentration, temperature reduction, cellular dehydration, freezing and thawing (Bailey *et al*., 2008). Each of these steps is potentially harmful to the functionality of the sperm, and each step adversely affects the movement pattern of those sperm surviving the process, since they exhibit more lineal and less vigorous motility immediately post thaw than do those examined during the cooling step (Eriksson *et al*., 2001).

Boar sperm is not well adapted to tolerate cooling to low temperatures. There is a reduction of their post-thaw viability and consequently their fertility, as a consequence of capacitation-like changes in their plasma membrane, as well as accumulated cellular injuries that arise throughout the cryopreservation process (Watson, 2000). Boar sperm are sensitive to peroxidative damage due to the relative high content of polyunsaturated fatty acids in the phospholipids of the membrane (Cerolini *et al*., 2001).

## 2.10 ARTIFICIAL INSEMINATION

Artificial insemination (AI) in pigs is common practice nowadays in all pig producing countries. In recent years, with the realisation of the potential value of reproductive techniques for pigs (when these techniques are optimised), more research has been done and use of AI in the industry is now growing rapidly (Weitze, 2000). According to Roca *et al*. (2006), even though cryopreservation of boar semen for AI was developed 35 years ago, cryopreservation conditions and AI strategies are still considered sub-optimal.

The use of frozen thawed sperm in AI technologies represents around 1% of total inseminations (Roca *et al*., 2006), mainly due to the poor reproductive output obtained with frozen–thawed sperm compared to refrigerated semen (Watson, 2000). The success of fertilization when frozen–thawed sperm doses used depends

not only on their quality or a proper timing between insemination and ovulation (Großfeld *et al*., 2008), but also on the site of sperm deposition (Ogbuewu *et al*. 2007).

Farrowing rates decrease by 20–30% and litter size drops by two to three piglets when using cryopreserved semen, in comparison to rates attained using liquid semen because approximately 40–50% of the sperm does not survive cryopreservation (Johnson, 1985). Although these differences in farrowing rates and litter size can be decreased or eliminated using deep intrauterine insemination or specialized sperm freezing methods (Eriksson *et al*, 2002; Roca *et al*, 2003), it is still generally accepted that cryopreserved sperm have a shorter window of optimum fertility relative to fresh extended/chilled sperm (Waberski *et al*., 1994b). This reduction in fertility of frozen thawed sperm is believed to be the result of cryoinjury to the sperm acquired during the freezing and thawing process (Bailey *et al*., 2000).

## 2.11 ASSESSMENT OF KINEMATICS AND MOTILITY OF SPERM

Computer Assisted Semen Analysis (CASA) is mostly for assessment of sperm concentration and specific patterns of sperm motility (velocity, linearity etc**)**. The available clinical data show that the measurement obtained by CASA are correlated with conception in vivo and fertilization in vitro, but comprehensive quality control and quality assurance programs are necessary to ensure accuracy. Computer assessment of kinetics of sperm motility such as average sperm velocity, curvilinear velocity speed (VCL) (the average path velocity of the sperm head along its actual trajectory); straight line velocity (VSL) (the average path velocity of the sperm head along a straight line from its first to its last position), average path velocity (VAP) (the average velocity of the sperm head along its average trajectory), linearity (LIN) (the ratio between VSL and VCL), straightness (STR) (the ratio between VSL and VAP)and wobble (WOB). The width of the sperm head's trajectory value recorded was the mean amplitude of lateral head displacement (ALH, mm), the average value of the extreme side-to-side movement of the sperm head in each beat cycle. Finally, the frequency of the change in direction of the sperm head was recorded by means of the beat cross frequency (BCF, Hz), the frequency with which the actual sperm trajectory crosses the average path trajectory. For more detailed descriptions of

these parameters, Mortimer (1997). Overall, commercial computer systems provide objective assessment of sperm velocity and movement characteristics which are useful for research purpose (Table 2.4).

**Table 2.4** The ARC Sperm Class Analyzer® settings used to analyse sperm motility and velocity parameters

| **Parameters** | **Settings** |
|---|---|
| Contrast | 169 |
| Brightness (MHz) | 470 |
| Image/second | 50 |
| Optic | Ph- |
| Chamber | Coverslide |
| Scale | 10X |
| Particle size ($\mu m^2$) | 10<70 |
| Slow (µm/second) | <40 |
| Medium (µm/second) | <80 |
| Rapid (µm/second) | <120 |
| Progressivity (%) | 40% of straightness |
| Circular (%) | 50% of linearity |
| Connectivity | 11 |
| Velocity on the average path points | 7 |
| Number of images | 50 |

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# CHAPTER 3

# Comparative study on semen characteristics of Kolbroek and Large White boars following Computer Aided Sperm Analysis® (CASA®)

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## 3.1 Abstract

Consistent estimates of boar fertility potential from objective semen evaluation could be a valuable tool for boar selection. The objective of this study was to evaluate semen characteristics of Kolbroek and Large White boars following Computer Aided Sperm Analysis® (CASA®). Twelve ejaculates were collected separately from individual Kolbroek (n=4) and Large White (n=4) boars using the gloved-hand technique. Following semen collection, semen was evaluated for macroscopic and microscopic characteristics. Analysis of variance (ANOVA) was used to test the differences between the breeds ($P<0.05$). The bodyweight of Kolbroek (154.7±8.5) was significantly lower compared to Large White (189.9±7.7) boar. There was also a positive correlation between bodyweight and semen volume of both Kolbroek ($r=0.2197$) and Large White ($r=0.2577$) boar. However, no significant differences were observed in Kolbroek and Large White boar semen volume (140 and 170 ml), sperm concentration (0.727 and 0.761 x $10^9$ sperm/ml), pH (7.0 and 7.0), total motility (95 and 91%) and morphology (84 and 82 %). In conclusion, the bodyweight of Kolbroek and Large White boar was positively correlated with ejaculated semen volume. Sperm characteristics of both Kolbroek and Large White boar were similar.

Sperm Class Analyser® provided a precise and objective more information of sperm motility characteristics.

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**Keywords: Sperm, Large White, Kolbroek, motility rate, boar**

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## 3.2 INTRODUCTION

Large White is the most popular exotic breed in South Africa (Agricultural Research Council, 1993) due to their superior fertility and growth rate (Ncube *et al*., 2003). However, their high nutrient requirements and intensive management systems make them unsuitable for resource-poor rural farmers and harsh environmental conditions. Kolbroek is a South African indigenous pig breed with unique genetic traits for diseases tolerance and adaptability in harsh environmental conditions (Ramsay *et al*., 1994). They are considered appropriate breed for the resource-poor rural farmers because of their tolerance to various diseases and capacity to utilize fibrous and poor quality feed resources compared to exotic breeds (Halimani *et al*., 2010).

A recent survey indicated a catastrophic collapse in the population of South African indigenous germplasm (Food and Agricultural Organization, 2007). This collapse was attributed among others to unplanned breeding, crossbreeding and introduction of exotic germplasm (Scholtz, 2005). Mating and crossbreeding are largely unsupervised leaving these breeds vulnerable to inbreeding and uncontrolled genetic admixture with other breeds (Halimani *et al*., 2010). Most of the research has been focused on the imported genotypes which cannot be sustained under smallholder conditions (Ncube *et al*., 2003). Hence there is a need to evaluate reproductive potential of imported boars in comparison with South African indigenous boars.

The reproductive potential of the indigenous Kolbroek boars has not been fully exploited in South Africa compared to other pig genotypes. A proper semen analysis is empirical for boar selection in the herd and for preserving their genetic materials through *ex-situ* and *in-situ.* Indigenous pigs were long regarded as unsuitable for intensive commercial breeding because of their slow growth and inadequate meat

production (Prolit, 2004). However, indigenous pigs exhibit well-established adaptations to severe environmental and management conditions (Swart *et al*., 2010). Moreover, there is lack of accurate method of predicting the fertility rate of Kolbroek boar sperm to determine their reproductive potential.

Sperm motility is known to be an important characteristic in predicting the fertility of male potential performances (Holt *et al*., 1997; Tardif *et al*., 1999; Gadea, 2005). However, subjective microscope evaluation varied between 30 to 60% from the same ejaculates (Amann, 1989). Due to these biases, emphasis has been placed on the use of objective methods such as Computer Aided Sperm Analysis® (CASA®) system (Saikhun *et al*., 2011). Therefore, the objective of this study was to compare South African indigenous Kolbroek and exotic Large White boar breeds on sperm characteristics following analysis by Computer Aided Sperm Analysis® (CASA®) known as Sperm Class Analyser® (SCA®).

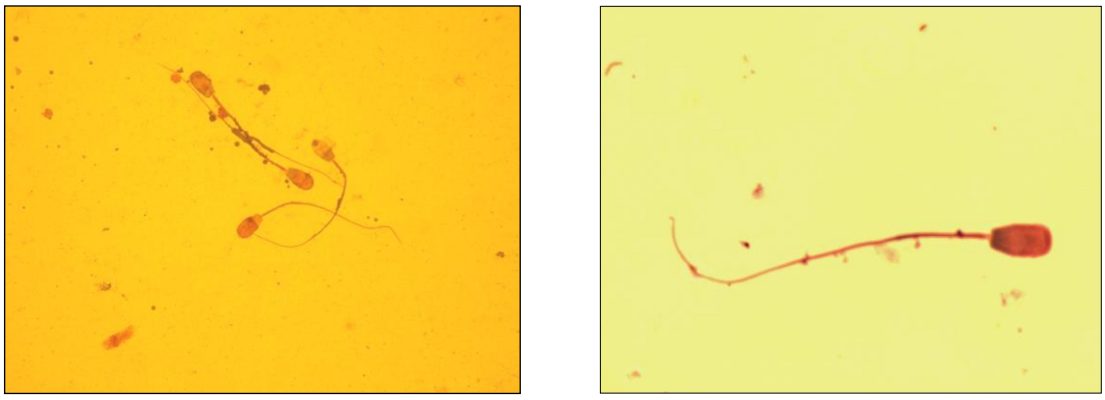
## 3.3 MATERIALS AND METHODS

The study was conducted at the Pig Research Unit of Agricultural Research Council (Germplasm Conservation & Reproductive Biotechnologies Unit), Irene, South Africa. The Agricultural Research Council-Irene campus is located at 25° 55' South; 28° 12' East. The institute is located in the Highveld region of South Africa and situated at an altitude of 1525m above sea level. Four indigenous Kolbroek and four exotic Large White boars were used for this study because of the scarcity challenge of finding Kolbroek boars. The boars were aged between two to four years of age. The study was done in summer season (February to March, 2011). The boars were weighed using a KM3 electronic weight indicator (Rudoweigh®). The boars were in good health condition throughout the duration of the study. The diets were formulated to meet the nutritional requirements of the boars (National Research Council (NRC), 1998). Water was given *ad libitum* throughout the duration of the study.

Semen samples were collected from the experimental boars twice weekly from February to March. Twelve ejaculates were collected separately from four Kolbroek and four Large White boars with the gloved-hand technique in a 300 ml glass beaker. The filtered semen fraction were sealed with a gauze filter inside a pre-

warmed (39ºC) insulated thermos flask. Upon arrival at the laboratory, semen volume was measured by using the graduated falcon tube, pH was measured using the litmus paper, and sperm concentration was measured using the spectrophotometer (Jenway 6310 spectrophotometer, Bibby Scientific, England) and was recorded in billions (x $10^9$/ml). Experimental boars were cared for according to the guidelines for the Agricultural Research Council, Animal Production Institute ethics committee (Ref: APIEC10/01).

Semen was collected from Kolbroek and Large White boars and a 10x dilution was prepared by adding semen to 0.9 % sodium chloride. One drop of 0.27 % Chicago Sky Blue and one drop of diluted semen were mixed on a slide. Slides were air-dried in a near vertical position then put into a fixative in a jar for two minutes, and then rinsed with tap and distilled water. Slides were put into jars containing the Giemsa staining solution and left for 20 hours at room temperature. Slides were rinsed again in tap and distilled water for two minutes, air-dried in a near vertical position and cover slipped with Methyl Yellow. A drop of oil immersion (Olympus, Japan) was placed on the smeared microscope glass slide and 100 sperm were counted at 100x magnification (**Figure 1A and B**). A criterion was applied for the evaluation for abnormal sperm head (flat, sharp, double and if it is not oval); midpiece (proximal and distal cytoplasmic droplets); tail (coiled, double, broken). A live sperm was white/pink in colour and a dead sperm was dark blue (Kovács & Foote, 1992).



**A** **B**

**Figure 3.1 (A)**: Kolbroek live sperm; **(B)** Kolbroek dead sperm

The 10 µl of raw semen were placed into 500 µl of BO wash medium in a 15 ml tube (Falcon® 352099, USA). The tube was then kept in $CO_2$ incubator (Sanyo, Japan) adjusted to 39°C. Five µl of semen was placed on the warm glass slide (~76 x 26 x 1mm, Germany) and placed with a warmed cover slip (22 x 22 mm, Germany) over the microscope-warm plate (Omron) adjusted at 39°C. The sperm motility rates were evaluated by Computer Aided Sperm Analysis system (Sperm Class Analyzer® [SCA] 5.0, Microptic, Barcelona, Spain) at the magnification of 10x (Nikon, China). The kinematic values recorded for each sperm included, in addition to the overall percentage of motile sperm, the velocity of movement, the width of the sperm head's trajectory, and the frequency of the change in direction of the sperm head (**Table 3.1**).

**Table 3.1** Sperm Class Analyzer® settings used to analyse sperm motility and velocity characteristics

| **Characteristics** | **Settings** |
|---|---|
| Contrast | 169 |
| Brightness | 470 |
| Image/second | 50 |
| Optic | Ph- |
| Chamber | Cover slide |
| Scale | 10X |
| Particle size ($\mu m^2$) | 10<70 |
| Slow (µm/second) | <40 |
| Medium (µm/second) | <80 |
| Rapid (µm/second) | <120 |
| Progressivity (%) | 40 % of straightness |
| Circular (%) | 50 % of linearity |
| Connectivity | 11 |
| Velocity on the average path points | 7 |
| Number of images | 50 |

The analysis was done using Genstat Software. The experiment was designed as a completely randomised design with two treatments (Kolbroek and Large White boars). Analysis of variance (ANOVA) was used to test for differences between the treatments. The data were acceptably normal with homogeneous treatment variances. Treatment means were separated using Fisher's protected t-test least significant difference (LSD) at a significant level of $P<0.05$ (Snedecor & Cochran, 1980). The correlation of the bodyweight with semen volume, concentration and sperm motility was performed using SAS statistical software. The Pearson two-sided was used to determine the correlation between bodyweight and the variables (Snedecor & Cochran, 1980).

## 3.4 RESULTS

The results of macroscopic evaluations are outlined in Table 3.2. The bodyweight of Kolbroek (154.7±8.5 kg) was significantly lower compared to Large White (189.9±7.7 kg) boar. There was a positive correlation between bodyweight and semen volume of Kolbroek (r= 0.2197) and Large White (r= 0.2577). Conversely, there was a negative correlation between bodyweight and sperm motility rate (r= -0.9655) and concentration (r= -0.6600) of Kolbroek. However, the bodyweight of Large White was positively correlated with sperm concentration (r= 0.3721) but negatively correlated to total motility (r=-0.1043). No significant differences were observed in Kolbroek and Large White boar volume (140 and 170 ml), semen pH (7.0 and 7.0) and sperm concentration (0.727 and 0.761 x $10^9$ sperm/ml). Furthermore, no individual variation was observed for semen volume, pH and concentration.

**Table 3.2** Macroscopic evaluation for Kolbroek and Large White boar semen (mean±SD)

| Breed | Bodyweight (kg) | Semen volume (ml) | Semen pH | Semen concentration (x$10^9$ sperm/ml) |
|---|---|---|---|---|
| **Kolbroek 1** | 166.5 | 130.0±26.5 | 7.0±0.0 | 0.533.4±90.8 |
| **Kolbroek 2** | 150.5 | 126.7±11.6 | 7.0±0.0 | 1.0521±283.3 |
| **Kolbroek 3** | 147.0 | 100.0±0.0 | 7.0±0.0 | 0.9073±333.1 |
| **Kolbroek 4** | 155.0 | 205.0±37.8 | 7.0±0.0 | 0.4153±174.2 |
| **Averages** | 154.7±8.5[a] | 140.4±48.6 | 7.0 | 0.727±340.8 |
| **Large White 1** | 196.6 | 226.7±100.2 | 7.0±0.0 | 0.646.7±82.2 |
| **Large White 2** | 190.6 | 145.0±42.7 | 7.0±0.0 | 0.605.0±328.0 |
| **Large White 3** | 179.0 | 180.0±45.8 | 7.0±0.0 | 0.590.3±135.9 |
| **Large White 4** | 193.4 | 158.3±18.9 | 7.0±0.0 | 1.203.4±487.9 |
| **Averages** | 189.9±7.7[b] | 177.5±60.4 | 7.0 | 0.761. 0±372.8 |

[ab] Different letters indicate significant differences within columns (P<0.05).

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The results for Kolbroek and Large White sperm morphology are presented in Table 3.3. The average percentage (±SD) of Kolbroek and Large White live sperm was 84.6±6.1 and 81.7±7.1% respectively. There was no significant differences ($P<0.05$) in abnormal sperm morphology of Kolbroek and Large White.

**Table 3.3** Sperm morphology and viability for Kolbroek and Large White boar semen (mean±SD)

| Breed | Live (%) | Dead (%) | Abnormalities (%) | | |
|---|---|---|---|---|---|
| | | | Head | Midpiece | Tail |
| **Kolbroek 1** | 88.7±3.1 | 9.7±4.0 | 0.7±1.2 | 0.0±0.0 | 1.0±1.7 |
| **Kolbroek 2** | 84.7±7.5 | 10.0±8.7 | 2.3±0.6 | 1.7±2.9 | 1.3±1.2 |
| **Kolbroek 3** | 82.0±1.0 | 13.3±6.0 | 2.7±3.8 | 1.0±1.7 | 1.0±1.7 |
| **Kolbroek 4** | 83.0±10.0 | 9.7±5.5 | 2.3±2.3 | 1.0±1.7 | 4.0±3.5 |
| **Averages** | 84.6±6.1 | 10.7±5.6 | 2.0±2.1 | 0.9±1.7 | 1.8±2.3 |
| **Large White 1** | 82.0±9.6 | 7.0±5.3 | 0.7±0.6 | 0.3±0.6 | 6.3±6.0 |
| **Large White 2** | 87.7±6.7 | 5.3±1.5 | 2.7±1.5 | 1.0±1.0 | 6.0±6.0 |
| **Large White 3** | 80.7±5.5 | 9.3±5.0 | 1.7±2.1 | 0.0±0.0 | 5.7±7.4 |
| **Large White 4** | 76.3±3.5 | 17.3±7.0 | 0.7±0.6 | 1.3±1.2 | 8.0±4.4 |
| **Averages** | 81.7±7.1 | 9.8±6.3 | 1.4±1.4 | 0.7±0.9 | 6.5±5.2 |



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The results of both Kolbroek and Large White sperm motility are presented in Table 3.4. The average percentage (±SD) of Kolbroek and Large White sperm motility was 95.2±4.2 and 91.4±6.2%, respectively. However, a significant difference was observed for rapid sperm motility of Kolbroek 4 (79.4±2.6%) as compared to all the other boars including Large White. No significant difference was observed for all other sperm motility and velocity characteristics for Kolbroek and Large White boar.



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**Table 3.4** Sperm motility and velocity rates for Kolbroek and Large White boars (mean±SD)

| Boars | Sperm motility | | | Sperm velocity | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | TM (%) | RAP (%) | PM (%) | VCL (µm/sec) | VSL (µm/sec) | VAP (µm/sec) | LIN (%) | STR (%) | WOB (%) |
| **Kolbroek 1** | 91.8±6.6 [a] | 35.5±1.2 [b] | 36.3±25.8 [a] | 135.2±31.1 [a] | 42.8±21.2 [a] | 82.7±12.4 [a] | 33.8±20.8 [a] | 51.4±21.8 [a] | 64.4±21.2 [a] |
| **Kolbroek 2** | 96.2±3.4 [a] | 54.0±8.0 [b] | 31.1±4.6 [a] | 133.6±12.1 [a] | 39.4±5.8 [a] | 97.7±18.9 [a] | 29.6±4.7 [a] | 40.8±5.1 [a] | 72.9±9.3 [a] |
| **Kolbroek 3** | 96.6±2.6 [a] | 52.1±9.9 [b] | 31.3±9.9 [a] | 132.7±1.9 [a] | 35.5±3.0 [a] | 84.0±20.9 [a] | 26.7±2.0 [a] | 43.3±6.4 [a] | 63.2±14.8 [a] |
| **Kolbroek 4** | 96.1±3.5 [a] | 79.4±2.6[a] | 48.7±13.3 [a] | 171.7±11.2 [a] | 46.0±5.4 [a] | 98.0±12.9 [a] | 26.8±2.2 [a] | 47.7±9.3 [a] | 57.1±7.0 [a] |
| **Averages** | 95.2±4.2 | 55.2±17.3 | 36.8±15.2 | 143.3±22.8 | 40.9±10.6 | 90.6±16.1 | 29.2±9.7 | 45.8±11.5 | 64.4±13.5 |
| **Large White 1** | 94.8±5.2 [a] | 46.4±21.2 [b] | 27.9±8.2 [a] | 136.2±37.2 [a] | 36.0±9.8 [a] | 86.8±27.3 [a] | 26.8±5.4 [a] | 42.3±9.0 [a] | 63.3±2.5 [a] |
| **Large White 2** | 87.2±10.6 [a] | 39.2±1.0 [b] | 15.3±3.6 [a] | 121.2±11.3 [a] | 27.7±1.0 [a] | 77.7±2.6 [a] | 23.0±3.1 [a] | 35.7±2.5 [a] | 64.4±4.1 [a] |
| **Large White 3** | 93.5±2.9 [a] | 38.3±11.8 [b] | 24.4±2.6 [a] | 121.2±16.0 [a] | 33.8±5.6 [a] | 73.3±10.5 [a] | 28.6±8.8 [a] | 47.6±15.6 [a] | 60.4±2.1 [a] |
| **Large White 4** | 89.9±3.7 [a] | 44.0±18.1 [b] | 23.6±6.6 [a] | 137.7±33.3 [a] | 33.8±8.9 [a] | 86.7±25.7 [a] | 24.5±1.2 [a] | 39.4±1.7 [a] | 62.3±5.1 [a] |
| **Averages** | 91.4±6.2 | 42.0±13.4 | 22.8±6.8 | 129.1±24.8 | 32.8±7.0 | 81.1±17.7 | 25.7±5.1 | 41.2±9.0 | 62.6±34 |

TM (total motility), RAP (Rapid), PM (progressive motility), VCL (velocity on the curve line), VSL (velocity on the straight line), VAP (velocity on the average path), LIN (linearity), STR (straightness), WOB (wobble). [ab]Different letters indicate significant differences (P<0.05).



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## 3.5 DISCUSSION

This study demonstrated that the bodyweight of Kolbroek (154.7±8.5) was significantly lower compared to Large White (189.9±7.7) boar. There was also a bodyweight correlation to semen volume ejaculated by both Kolbroek (r=0.2197) and Large White (r=0.2577). However, no significant differences were observed for Kolbroek and Large White boar semen volume (140 and 170 ml), sperm concentration (0.727 and 0.761 x $10^9$ sperm/ml), pH (7.0 and 7.0), total motility rate (95 and 91%) and morphology (84 and 82 %). Similarly, it was previously reported that breed did not have a significant effect on boar sperm characteristics (Kennedy & Wilkins, 1984; Rothschild, 1996; Oh *et al*., 2003). Kolbroek boars had a slightly lower semen volume as compared to the standard semen volume of 150 to 300 ml in exotic breeds (Kondracki, 2003). Egerszegi *et al*. (2008) reported similar results for Hungarian indigenous Mangalica boars (178 ml). In contrast Wolf & Smithal. (2009) found that Czech Large White and Landrace had a slightly higher semen volume of 276 and 273 ml, respectively.

Chimonyo *et al*. (2005) reported that indigenous pigs in southern Africa are smaller in size compared to exotic pig breeds. This was evident in the present study as Kolbroek boars had a lower bodyweight (154.8 kg) as compared to Large White boar (189.9 kg). Larger breeds such as Large White tended to produce higher semen volume (Hughes & Varely, 1980). Although Kolbroek boar bodyweight was lower, the sperm concentration was higher. There was a negative correlation between bodyweight and sperm motility rate (r= -0.9655) and concentration (r= -0.6600); but positively correlated with volume (r= 0.2197) of Kolbroek boar. However, the bodyweight of Large White was positively correlated with volume (r= 0.2577) and sperm concentration (r= 0.3721) but negatively correlated to total motility (r=-0.1043).

Johnson *et al*. (2000) reported that the pH of raw boar semen varies between 7.0 and 7.5, irrespective of the boar breed. This is in agreement with the present observed pH results (7.0) in both breeds. However, a pH change (increase or decrease) is detrimental to both the sperm metabolism and


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motility. Infection is usually associated with alkaline ejaculate (pH>8.0) which leads to diminished sperm motility and an increased proportion of altered acrosomes (Althouse *et al*., 2000). In the present study, the boar semen pH did not negatively affect the sperm motility.

No differences were observed for Kolbroek and Large White boar sperm concentration. Variation in the number of sperm in an ejaculate has been described between different pig breeds (Kommisrud *et al*., 2002), which is a first factor influencing semen dose production. Not only differences in sperm concentration but also in sperm volume (Kondracki, 2003), influence sperm concentration. The sperm concentration for indigenous Kolbroek was higher ($0.727 \times 10^9$ sperm/ml) as compared to the Hungarian Mangalica boar ($0.490 \times 10^9$ sperm/ml) (Egerszegi *et al*., 2008).

The percentage of sperm with normal morphology was above 80% for Kolbroek and Large White boars. Such percentages of normal morphology are correlated with fertility (Sanchez *et al*., 1998; Xu *et al*., 1998; Alm *et al*., 2006). The results from this study also showed that there are no variations between individual boars, irrespective of the breed. Similar findings were observed where Borg *et al*. (1993) reported that characteristics of sperm morphology did not differ among different boar breeds (Duroc, Meishan, Fengjing and Minzhu boars). Kolbroek and Large White semen showed a lower percentage of morphologically abnormal sperm (4.7±2.0% and 2.9±2.5%, respectively) as compared to other authors. Wolf & Smithal (2009) found a slightly higher percentage of abnormal sperm (11.4 and 11.2%) for Czech Large White and Czech Landrace boars, respectively. Criteria for the maximum percentage of primary and secondary abnormalities in commercial pig AI-centres were determined as 10% and 20%, respectively (Waberski *et al*., 1994; Flowers, 1997). Morphological abnormalities give an indication of aberrations in the spermatogenesis. Morphological abnormalities of sperm can have a detrimental impact upon fertilization and embryonic development (Walters *et al*., 2005; Saacke, 2008).



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The average sperm total motility obtained for Kolbroek and Large White was 95 and 91%, respectively. These sperm motility results are an indication of an active metabolism and are considered to be of great importance for fertilizing to take place. Lower motility percentages were reported (70.2±8.8%) for Czech hybrid AI boars (Frydrychová *et al*., 2010). Subjective method was used to evaluate sperm motility analysis. Microscopic techniques have limitations including subjectivity, variability, the small number of sperm analysed and poor correlation with fertilizing potential (Rijsselaere *et al*., 2005). Subjective visual evaluation of motility is also prone to human error and biasness. Hence, the computer-assisted sperm analysis® (CASA®) was initiated to reduce subjective bias on the motility assessment and to discriminate a series of motility patterns of boar semen (Tretipskul *et al*., 2010).

## 3.6 CONCLUSION

The bodyweight of Kolbroek and Large White boar was positively correlated with ejaculated semen volume. However, macroscopic and microscopic sperm characteristics of Kolbroek were similar compared to Large White boar. Surprisingly, Kolbroek boar sperm concentration and motility rate was negatively correlated to bodyweight compared to only Large White sperm concentration. This is the first study that provided more information on sperm motility characteristics of both Kolbroek and Large White boar using Sperm Class Analyser®. It is recommended that further studies should be conducted with more number of boars to validate the sperm motility characteristics information following artificial insemination.

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# CHAPTER 4

**Effect of *in vitro* storage temperature on Kolbroek and Large White boar sperm motility rate using Computer Aided Sperm Analysis®**

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## 4.1 Abstract

Increasing use of artificial insemination (AI) in the pig industry emphasizes the need for distribution of good quality semen. The objective of this study was to find the suitable storage temperature (17 vs. 25$^{o}$C) on sperm motility rate of Kolbroek and Large White boar following Computer Aided Sperm Analysis®. Twelve ejaculates were collected separately from individual Kolbroek (n=4) and Large White (n=4) boars using the gloved-hand technique. Following semen collection, the semen was diluted (1:1 v/v) and evaluated by Sperm Class Analyzer® (SCA®) at 0, three, six and 24 hours interval. The data were analysed using analysis of variance with SAS statistical software. A higher proportion of sperm motility rate of Kolbroek (78.2%) and Large White (79.3%) boar was maintained when stored at 17°C for 24 hours. Sperm motility rate of Kolbroek boar (61.0%) at 25°C was affected by temperature. However, prolonged storage of semen did not influence Large White boar sperm motility rate, irrespective of the temperature. In conclusion, temperature did not affect Kolbroek and Large White boar sperm motility rate. However, storage of Kolbroek boar semen at 25°C resulted in a slightly lower total sperm motility rate.

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**Keywords: Boar semen, Large White, Kolbroek, CASA**
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## 4.2 INTRODUCTION

Artificial insemination (AI) is one of the assisted reproductive technologies (ART) developments that have made a major contribution to both animal improvement and the conservation of farm animal genetic resources. However, successful application of AI technique requires identification of a suitable semen extender and temperature. Liquid preservation is still a preferred method of boar semen storage (Weitze, 1991) when diluted semen is stored at 15-20°C for several days before it used for artificial insemination (Johnson *et al*., 2000). In practice, semen is collected in isolated cans to avoid contact with colder surfaces and subsequent dilution is done with extender at 35-37°C (Althouse, 1997) after which the semen is allowed to cool down gradually to 15-20°C where the sperm acquire a gradual resistance to cold shock (Althouse, 1997). Further storage of diluted semen is done at 17°C, at which temperature semen metabolism is reduced (Althouse *et al*., 1998; Paulenz *et al*., 1998), a condition necessary to extend storage time (Johnson *et al*., 2000). A gradual reduction in the metabolic activity of boar spermatozoa during storage at cold shock temperature could limit the production of detrimental by-products, which might compromise sperm function (Althouse *et al*., 1998). Moreover, there is lack of accurate method of predicting the fertility rate of Kolbroek boar sperm to determine their reproductive potential.

The ultimate goal for semen analysis is to accurately, objectively, rapidly and inexpensively predict the fertility of a sample (Saikhun *et al*., 2011). Microscopic techniques have limitations including subjectivity, variability, the small number of sperm analyzed and poor correlation with fertilizing potential (Rijsselaere *et al*., 2005). Computer-assisted sperm analysis (CASA) was initiated to reduce subjective biasness on motility rate assessment and to discriminate a series of motility patterns of boar sperm (Tretipskul *et al*.,

2010). The objective of this study was to find the suitable storage time and equilibration temperature (17 vs. 25$^{o}$C) for boar sperm using Class Analyzer®.

## 4.3 MATERIALS AND METHODS

The study was conducted at the Pig Research Unit of Agricultural Research Council (Germplasm Conservation & Reproductive Biotechnologies Unit), Irene, South Africa. The Agricultural Research Council-Irene campus is located at 25° 55' South; 28° 12' East. The institute is located in the Highveld region of South Africa and situated at an altitude of 1525m above sea level. Four indigenous Kolbroek and four exotic Large White boars were used for this study because of the scarcity challenge of finding Kolbroek boars. The boars were aged between two to four years of age. The diets were formulated to meet the nutritional requirements of the boars (NRC, 1998). Water was given *ad libitum* throughout the duration of the study. Experimental animal were cared for according to the guidelines for the Agricultural Research Council, Animal Production Institute ethics committee (Ref: APIEC10/01).

Semen samples were collected from the experimental boars twice weekly. Twelve ejaculates were collected separately from the eight boars, namely four Kolbroek and four Large White boar breeds with the gloved-hand technique in a 300 ml glass beaker. The filtered sperm fraction were sealed with a gauze filter inside a pre-warmed (39ºC) insulated thermos flask. Upon arrival at the laboratory, semen was evaluated for microscopic characteristics; sperm motility using Sperm Class Analyzer® (SCA®). Semen was then extended with Beltsville Thawing Solution (BTS) extender in 50 ml disposable centrifuge tubes at a ratio of 1:1 (v/v) and stored at 17ºC and 25ºC. Then the semen samples were evaluated for sperm motility and velocity at three, six and 24 hour intervals using SCA®.

The 10 µl of raw semen was placed into 500 µl of swim up medium in a 15 ml tube (Falcon® 352099, USA). The tube was then kept in $CO_2$ incubator (Sanyo, Japan) adjusted to 39°C. Five µl of semen was placed on the warm glass slide (~76 x 26 x 1mm, Germany) and placed with a warmed cover slip

(22 x 22 mm, Germany) over the microscope-warm plate (Omron) adjusted at 39°C. The sperm motility parameters were evaluated by Computer Aided Sperm Analysis system (Sperm Class Analyser® 5.0, Microptic, Barcelona, Spain) at the magnification of 10x (Nikon, China). Before the track sequence was to be analysed, the trajectory of each sperm identified and recorded in each field was visually assessed to eliminate possible debris and to diminish the risk that unclear tracks were included in the analyses. The settings are shown on Table 4.1.

The appropriate analysis of variance (ANOVA) was performed on the data using SAS statistical software. Shapiro-Wilk test was performed to standardise residuals to test for deviations from normality student t-LSD was calculated at the 5% level of significance to compare means of significance difference. The treatment means with the same letter do not differ significantly.

**Table 4.1** The ARC Sperm Class Analyzer® settings used to analyse sperm motility and velocity parameters

| Parameters | Settings |
|---|---|
| Contrast | 169 |
| Brightness (MHz) | 470 |
| Image/second | 50 |
| Optic | Ph- |
| Chamber | Coverslide |
| Scale | 10X |
| Particle size ($\mu m^2$) | 10<70 |
| Slow (µm/second) | <40 |
| Medium (µm/second) | <80 |
| Rapid (µm/second) | <120 |
| Progressivity (%) | 40% of straightness |
| Circular (%) | 50% of linearity |
| Connectivity | 11 |
| Velocity on the average path points | 7 |
| Number of images | 50 |

## 4.4 RESULTS

The effect of *in vitro* storage temperature and period on Kolbroek and Large White boar sperm motility is shown in **Table 4.2**. The raw sperm motility rate Kolbroek (88.0±16.0%) and Large White (78.7±15.0%) declined as the period of storage was extended. The percentage of rapid motile sperm from Kolbroek semen samples were significantly lower (32.8±15.5%) as compared to sperm stored for a prolonged period, irrespective of the storage temperature. The rapid sperm motility rate for Kolbroek was higher at three, six and 24 hours irrespective of temperature, except for sperm stored at $25^0$C after 24 hours (18.0±13.8%). The percentage of rapid motile sperm from Large White semen samples were significantly higher (52.0±23.0%) when stored at 17°C for six

hours. The proportion of progressively motile sperm was low (>40%) throughout the storage period for both Kolbroek and Large White boars.

The effect of *in vitro* storage temperature and period on Kolbroek and large White boar sperm velocity is shown in Table 4.3. No significant differences were observed in both Kolbroek and Large White sperm velocity rates, irrespective of temperature and time. Kolbroek and Large White boar sperm showed an active type of movement (high VCL) but considerably reduced forward progression (lower VSL). The characteristics of Kolbroek and Large White boar sperm were consistent with erratic behaviour (high VCL, low VSL and low LIN) indicating that the extender exerted a subtle protective effect on the swimming behaviour. Thus this suggests that the storage temperature (17 and 25ºC) was able to maintain sperm velocity characteristics in a similar manner during storage. Furthermore, the Kolbroek and Large White sperm trajectories were irregular (VCL was much higher than VAP, and the LIN was very low) and showed a high degree of lateral deviation of the head from the direction of movement (low STR).

**Table 4.2.** *In vitro* storage temperature and period on Kolbroek and large White boar sperm motility (mean±SD).

| Boar breed | Temperature (°C) | Storage period (h) | TM (%) | RAP (%) | PM (%) | NPM (%) | SLO (%) | MED (%) |
|---|---|---|---|---|---|---|---|---|
| **Kolbroek** | **39°C** | Raw | 88.0±16.0$^{ab}$ | 32.8±15.5$^{b}$ | 26.9±8.9$^{bc}$ | 61.2±11.3$^{a}$ | 24.1±8.6$^{a}$ | 31.2±13.3$^{a}$ |
| | **17°C** | 3 | 82.3±11.8$^{ab}$ | 53.7±21.4$^{a}$ | 29.3±10.0$^{abc}$ | 53.1±8.7$^{abc}$ | 14.3±8.0$^{b}$ | 14.3±8.8$^{b}$ |
| | | 6 | 87.3±10.6$^{ab}$ | 50.3±25.9$^{a}$ | 38.3±17.9$^{a}$ | 49.0±14.9$^{bcd}$ | 14.8±7.1$^{b}$ | 20.9±17.1$^{ab}$ |
| | | 24 | 78.2±23.2$^{b}$ | 44.1±20.4$^{ab}$ | 35.4±15.4$^{ab}$ | 42.8±15.3$^{cd}$ | 17.3±8.5$^{b}$ | 16.8±9.3$^{b}$ |
| | **25°C** | 3 | 91.7±5.6$^{a}$ | 51.7±11.5$^{a}$ | 30.6±11.6$^{abc}$ | 61.1±12.1$^{a}$ | 20.0±6.7$^{ab}$ | 20.1±12.1$^{b}$ |
| | | 6 | 87.1±6.5$^{ab}$ | 49.7±17.9$^{a}$ | 30.8±9.1$^{abc}$ | 56.4±10.7$^{ab}$ | 15.0±6.3$^{b}$ | 22.5±14.6$^{ab}$ |
| | | 24 | 61.0±28.3$^{c}$ | 18.0±13.8$^{c}$ | 23.4±14.6$^{c}$ | 37.6±11.2$^{d}$ | 19.1±11.7$^{ab}$ | 23.8±12.5$^{ab}$ |
| **Large White** | **39°C** | Raw | 78.7±15.0$^{ab}$ | 34.6±20.6$^{b}$ | 29.8±17.5$^{bc}$ | 49.0±20.8$^{ab}$ | 29.5±13.7$^{ab}$ | 18.0±6.2$^{a}$ |
| | **17°C** | 3 | 86.3±7.8$^{a}$ | 42.8±17.8$^{ab}$ | 41.3±23.8$^{ab}$ | 45.1±21.9$^{ab}$ | 22.6±10.4$^{b}$ | 21.7±15.3$^{ab}$ |
| | | 6 | 85.5±13.1$^{ab}$ | 52.0±23.0$^{a}$ | 38.9±21.2$^{ab}$ | 46.6±22.4$^{ab}$ | 17.7±11.5$^{ab}$ | 15.7±9.7$^{b}$ |
| | | 24 | 79.3±14.2$^{ab}$ | 39.3±24.1$^{ab}$ | 36.3±20.3$^{abc}$ | 45.0±12.9$^{ab}$ | 18.6±12.3$^{ab}$ | 21.4±16.3$^{b}$ |
| | **25°C** | 3 | 82.9±14.3$^{ab}$ | 31.8±22.5$^{b}$ | 33.6±17.2$^{abc}$ | 49.3±23.9$^{a}$ | 25.9±16.9$^{ab}$ | 20.0±17.0$^{ab}$ |
| | | 6 | 78.0±14.4$^{b}$ | 31.4±19.5$^{b}$ | 25.6±13.9$^{c}$ | 52.4±18.3$^{a}$ | 25.5±14.1$^{a}$ | 23.0±16.5$^{ab}$ |
| | | 24 | 85.6±12.5$^{ab}$ | 40.2±25.4$^{ab}$ | 46.0±20.7$^{a}$ | 39.7±18.0$^{b}$ | 19.1±11.4$^{ab}$ | 24.8±21.0$^{b}$ |

TM (total motility); PM (progressive motility); NPM (non progressive motility); RAP (rapid motility); MED (medium motility) and SLO (slow motility). $^{abc}$Different letters indicate significant differences within columns ($P < 0.05$).

**Table 4.3.** *In vitro* storage temperature and period on Kolbroek boar sperm velocity (mean±SD).

| Breed | Temperature (°C) | Storage period (h) | VCL (µm/sec) | VSL (µm/sec) | VAP (µm/sec) | LIN (%) | STR (%) | WOB (%) |
|---|---|---|---|---|---|---|---|---|
| **Kolbroek** | **39°C** | Raw | 112.3±14.6$^{b}$ | 29.8±7.9$^{ab}$ | 65.7±13.6$^{b}$ | 26.8±7.7$^{a}$ | 45.0±5.4$^{bc}$ | 58.7±11.1$^{a}$ |
| | **17°C** | 3 | 157.9±36.0$^{a}$ | 32.1±7.5$^{ab}$ | 76.4±17.9$^{ab}$ | 20.7±3.7$^{bc}$ | 42.7±6.9$^{c}$ | 48.5±2.9$^{b}$ |
| | | 6 | 150.1±34.5$^{a}$ | 38.8±13.5$^{b}$ | 84.9±20.8$^{a}$ | 26.3±9.5$^{ab}$ | 46.0±11.6$^{bc}$ | 57.4±12.2$^{a}$ |
| | | 24 | 139.9±28.3$^{a}$ | 33.7±12.2$^{ab}$ | 66.0±14.9$^{b}$ | 24.2±8.5$^{abc}$ | 50.4±11.1$^{ab}$ | 47.1±6.2$^{b}$ |
| | **25°C** | 3 | 139.9±17.5$^{a}$ | 31.4±11.8$^{ab}$ | 68.9±16.4$^{b}$ | 20.3±2.9$^{c}$ | 42.1±7.2$^{c}$ | 49.2±8.9$^{b}$ |
| | | 6 | 148.6±26.7$^{a}$ | 31.9±7.6$^{ab}$ | 70.0±16.5$^{b}$ | 23.6±8.8$^{abc}$ | 43.6±9.3$^{bc}$ | 47.0±6.0$^{b}$ |
| | | 24 | 100.3±12.4$^{b}$ | 27.3±5.2$^{b}$ | 48.7±13.3$^{c}$ | 27.2±3.5$^{a}$ | 57.3±7.7$^{a}$ | 48.1±8.0$^{b}$ |
| **Large White** | **39°C** | Raw | 104.0±24.7$^{c}$ | 27.8±6.9$^{b}$ | 62.7±12.7$^{c}$ | 27.6±7.7$^{ab}$ | 45.4±12.1$^{c}$ | 61.0±2.0$^{a}$ |
| | **17°C** | 3 | 142.2±24.8$^{a}$ | 36.8±11.2$^{ab}$ | 82.1±13.7$^{a}$ | 26.2±12.1$^{ab}$ | 45.8±11.9$^{a}$ | 58.0±6.2$^{ab}$ |
| | | 6 | 146.4±27.0$^{a}$ | 36.8±11.8$^{ab}$ | 80.9±16.0$^{a}$ | 25.5±8.0$^{ab}$ | 42.6±9.6$^{a}$ | 55.3±4.2$^{bc}$ |
| | | 24 | 144.3±34.9$^{a}$ | 32.1±11.5$^{b}$ | 75.6±22.0$^{ab}$ | 22.2±5.7$^{b}$ | 50.6±16.4$^{a}$ | 52.8±4.9$^{c}$ |
| | **25°C** | 3 | 127.8±40.9$^{ab}$ | 37.0±22.5$^{ab}$ | 70.5±30.8$^{bc}$ | 32.3±14.3$^{a}$ | 44.6±12.5$^{a}$ | 57.5±10.8$^{ab}$ |
| | | 6 | 118.0±45.0$^{bc}$ | 40.9±25.8$^{ab}$ | 76.2±20.7$^{ab}$ | 27.2±8.1$^{ab}$ | 49.6±16.3$^{a}$ | 60.8±4.6$^{a}$ |
| | | 24 | 133.2±43.2$^{ab}$ | 53.0±45.7$^{a}$ | 75.7±28.7$^{ab}$ | 28.6±10.0$^{ab}$ | 45.8±11.9$^{a}$ | 56.5±6.3$^{bc}$ |

VCL (velocity on the curve line); VSL (velocity on the straight line); VAP (velocity on the average path); LIN (linearity); STR (straightness) and WOB (wobble). $^{abc}$Different letters indicate significant differences within columns ($P < 0.05$).

## 4.5 DISCUSSION

This study demonstrated that temperature and storage period did not affect sperm motility rate of Large White; however, sperm motility rate of Kolbroek boar was affected by temperature and storage period. It is a known fact that sperm originating from different breeds and individuals vary in many respects. Kolbroek boar raw sperm motility rate (88.0±16.0%) declined slightly at three hour storage (82.3±11.8%) due to exposure to cold shock which occurs when raw ejaculated boar semen are diluted and quickly cooled below body temperature (39°C). However, Kolbroek boar sperm motility was maintained after three hour storage at 25°C (91.7±5.6%). Kolbroek boar total sperm motility was maintained at three hour (82.3±11.8 and 91.7±5.6%) and six hour storage (87.3±10.6 and 87.1±6.5%) at 17 and 25°C, respectively. At this point the sperm acquired gradual resistance against cold shock. However, a drastic decline was observed for Kolbroek boar total sperm motility (61.0±28.3%) at 24 hour storage.

There was a decline in the motility rate of both Kolbroek and Large White boar semen stored at 17 and 25°C for 24 hours. This is in agreement with the previous studies which observed insignificant decreases in sperm motility rate of boar semen extended with BTS within 24 hours of storage at 15 to 18°C (Zou & Yang, 2000; Waberski *et al*., 1994). Storage period and temperature influenced Kolbroek sperm total motility (61.0%) and rapid motility (18.0±13.8%) at 25°C after 24 hours. These findings suggest that BTS is not a suitable extender for Kolbroek boar semen storage post 24 hours. Conversely, prolonged storage of Large White semen (85.6±12.5) maintained high sperm motility at 25°C after 24 hours compared to Kolbroek. Additionally, sperm motility of more than 60% is still considered fertile (Britt *et al*., 1999), hence the 61.0% attained for Kolbroek boar semen stored at 25°C for 24 hours may be considered optimal. Consequently Kolbroek and Large White semen do not need to be frozen if they will be used for artificial insemination within 24 hours.

Large White boars maintained a higher total sperm motility rate when stored at 17 (79.3±14.5%) and 25°C (85.6±12.5%) for 24 hours. In contrast, Zou &

Yang (2000) reported lower sperm total motility (41.7±5.2%) for Harbin White and Long White boar breeds for semen stored at 15°C after 24 hours. It was also reported that Hampshire boar sperm motility declined significantly from the initial motility of 87.50±0.97 to 66.25±1.76% after 24 hours at 18°C (Kumaresan *et al*., 2009). However, subjective analysis was used for these findings. Subjective visual evaluation of motility was prone to human error and biasness. This further suggests that sperm originating from different breeds vary in many respects.

Due to sensitivity of boar sperm to low temperatures, declines in storage temperature are likely to result in decreases in sperm motility; this phenomenon was observed in the present study. In order to maximize motility during liquid storage, the 17°C temperature must be maintained to avoid decreases in total motility and progressive motility as well as sperm velocities as indicated in the present study. Further storage of diluted semen done at 15-20°C reduces sperm metabolism (Althouse *et al*., 1998; Paulenz *et al*., 1998) a condition necessary to extend storage time (Johnson *et al*., 2000). However, boar sperm are sensitive to cold shock due to their physic-chemical characteristics (Verstegen *et al*., 2002), specifically the lipid composition of the plasma membrane. This susceptibility to cold shock makes it necessary to preserve semen samples at between 15 and 20°C (Paulenz *et al*., 2000; Kommisrud *et al*., 2002), to induce cold resistance (Paulenz *et al*., 2000). However, it was evident in this study that 25°C preserves boar sperm motility rate up to 24 hours regardless of breed.

The velocity characteristics, especially VCL, tended to increase during the three and six hours storage, irrespective of the breed. The low Linearity indicates that these sperm could have been either hyperactivated or showing erratic and uncoordinated movement, irrespective of the storage temperature, time and breed. The CASA® provided the objective assessment of the sperm characteristics (Verstegen *et al*., 2002). Reduced sperm motility may be due to consequences of cold shock during prolonged storage (Kumaresan *et al*., 2009), which was evident in the present study.

## 4.6 CONCLUSION

In conclusion, temperature and storage period did not affect the sperm motility of Large White; however, sperm motility of Kolbroek boar was affected by temperature and storage period. Kolbroek and Large White boar sperm stored at 18°C for 24 hours maintained the acceptable sperm motility rate. Thus, Kolbroek and Large White boar semen may be transported overnight to any South African Developing Countries when stored at 17°C within 24 hours for artificial insemination. The SCA® has a great potential for routine evaluation of boar semen and in the development of new semen liquid storage methods. It provides more precision and objectivity to the conventional assessment of sperm characteristics. However, future studies are required to determine suitable short term extender for indigenous Kolbroek boars for a period of more than 72 hours and linking CASA-SCA motility parameters with pregnancy and birth rate following AI.

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# CHAPTER 5

**Effect of extender and storage period on the South African indigenous Kolbroek and Large White boar sperm motility rate following analysis by Computer Aided Sperm Analysis®**

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## 5.1 Abstract

The choice of the short-term semen extender is important to preserve the diluted boar sperm before artificial insemination. The aim of this study was to identify suitable extender for short-term semen storage at 17°C of Kolbroek and Large White boars. Twelve ejaculates were collected separately from individual Kolbroek (n=4) and Large White (n=4) boars using the gloved-hand technique. Following semen collection, the semen was evaluated for macroscopic and microscopic characteristics. The semen was pooled and diluted with Beltsville Thawing Solution, Kobidil$^{+}$, Citrate or Tris-based extender. Sperm motility rate was evaluated using Sperm Class Analyzer® at 0, three, 24 and 48 hour interval. Data were analysed using analysis of variance. A positive correlation existed between bodyweight and semen volume for Kolbroek (r= 0.22) and Large White (r= 0.26). Higher sperm motility rate was observed in semen diluted with Tris-based extender for Kolbroek (74.1%) boar at 48 hours of storage. In contrast, sperm motility rate was higher for Large White boar semen diluted with BTS (62.9%), Kobidil (69.3%) and Tris (65.1%) compared to Citrate (27.6%) at 48 hour storage. In

conclusion, sperm motility rate was higher for Kolbroek boar semen diluted with Tris-based extender and Large White boar semen diluted with BTS, Kobidil and Tris-based extenders.

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Keywords: Semen, Kolbroek, period, extenders, indigenous
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## 5.2 INTRODUCTION

Artificial insemination (AI) during pig production is widely applied throughout the developed world by the use of semen that is preserved in the liquid state, which is stored at 15-20 °C for several days until it is used for AI (Johnson *et al*., 2000). To preserve spermatozoa for prolonged periods, their metabolic activity needs to be reduced by dilution into an appropriate extender and by lowering the temperature (Martín-Hidalgo *et al*, 2011). Storage and choice of extender are crucial for semen distribution and to optimize efficient use of semen for AI. In *vivo*, sperm are diluted with seminal fluids from the accessory glands at ejaculation and their motility is retained for a few hours (Johnson *et al*., 2000). To extend their survival *in vitro*, it is necessary to reduce the metabolic activity by chemical inhibitors or by lowering the temperature, which also requires dilution (Johnson *et al*., 2000).

During storage, boar spermatozoa undergo several changes, including diminished motility and viability and alterations in membrane permeability (Waterhouse *et al*., 2004). In addition, boar spermatozoa are susceptible to cold shock. This may be related to the lipid composition of the boar sperm membrane, which contains a high concentration of polyunsaturated fatty acids (Kumaresan *et al*., 2008). Beltsville Thawing Solution (BTS) is a commercial short-term boar semen extender, and has shown consistent fertility results after artificial insemination with semen stored for up to three days (Hofmo, 2000). The level of damage due to the liquid preserved boar sperm is commonly assessed by indicators of viability including sperm motility.

Different characteristics can be used to evaluate the quality of boar semen. Sperm motility is an indication of an active metabolism and the integrity of membranes (Johnson *et al*, 2000) and is considered to be of great importance for fertilizing. Due to this biasness, emphasis has been placed on the use of objective methods such as Computer Aided Sperm Analysis® (CASA®) system (Saikhun *et al*., 2011). The objective of this study was to identify the suitable extender for short term Kolbroek boar semen storage at 17°C following analysis by Computer Aided Sperm Analysis®.

## 5.3 MATERIALS AND METHODS

The study was conducted at the Pig Research Unit of Agricultural Research Council (Germplasm Conservation & Reproductive Biotechnologies Unit), Irene, South Africa. The Agricultural Research Council-Irene campus is located at 25° 55' South; 28° 12' East. The institute is located in the Highveld region of South Africa and situated at an altitude of 1525m above sea level. Four indigenous Kolbroek and four exotic Large White boars were used for this study because of the scarcity challenge of finding Kolbroek boars. The boars were aged between two to four years of age. The diets were formulated to meet and exceed the nutritional requirements of the boars (NRC, 1998). Water was given *ad libitum* throughout the duration of the study. Experimental animal were cared for according to the guidelines for the Agricultural Research Council, Animal Production Institute ethics committee (Ref: APIEC10/01).

Semen samples were collected from the experimental boars twice weekly. Twelve ejaculates were collected separately from the eight boars, namely four Kolbroek and four Large White boar breeds with the gloved-hand technique in a 300 mL glass beaker. The filtered sperm fraction were sealed with a gauze filter inside a pre-warmed (39ºC) insulated thermos flask. Upon arrival at the laboratory, semen was evaluated for microscopic characteristics; sperm motility using Sperm Class Analyzer® (SCA®). The semen was pooled and diluted with four different short term extenders, namely: BTS, Kobidil$^{+}$, Citrate and Tris-based extenders (Table 5.1) at a ratio of 1:1 (v/v). Sperm motility

characteristics were evaluated using SCA® at 0, three, 24 and 48 hours interval.

**Table 5.1** Composition (g/L) of different extenders for short-term liquid storage

| **Composition (g/L)** | **BTS** (Pursel & Johnson, 1975) | **Citrate** | **Tris** | **Kobidil** |
|---|---|---|---|---|
| Glucose | 37.00 | 10.00 | 10.00 | 37.00 |
| EDTA | 1.25 | - | - | 1.25 |
| Sodium Citrate | 6.00 | 18.56 | - | 6.00 |
| Tris | - | - | 24.22 | - |
| Citric Acid | - | - | 13.60 | - |
| Sodium Bicarbonate | 1.25 | - | - | 1.25 |
| Potassium Chloride | 0.75 | - | - | 0.75 |
| Gentamycin | - | 1.00 | 1.00 | 2.00 |

BTS: Beltsville Thawing Solution; EDTA: Ethylenediaminetetraacetic Acid

The 10 µl of raw semen was placed into 500 µl of swim up medium in a 15 ml tube (Falcon® 352099, USA). The tube was then kept in $CO_2$ incubator (Sanyo, Japan) adjusted to 39°C. Five µl of semen was placed on the warm glass slide (~76 x 26 x 1mm, Germany) and placed with a warmed cover slip (22 x 22 mm, Germany) over the microscope-warm plate (Omron) adjusted at 39°C. The sperm motility characteristics were evaluated by Computer Aided Sperm Analysis system (Sperm Class Analyser® 5.0, Microptic, Barcelona, Spain) at the magnification of 10x (Nikon, China). Before the track sequence was to be analysed, the trajectory of each sperm identified and recorded in each field was visually assessed to eliminate possible debris and to diminish the risk that unclear tracks were included in the analyses. The settings are shown on Table 5.2.

**Table 5.2** The ARC Sperm Class Analyzer® settings used to analyse sperm motility and velocity characteristics

| Characteristics | Settings |
|---|---|
| Contrast | 169 |
| Brightness (MHz) | 470 |
| Image/second | 50 |
| Optic | Ph- |
| Chamber | Coverslide |
| Scale | 10X |
| Particle size ($\mu m^2$) | 10<70 |
| Slow (µm/second) | <40 |
| Medium (µm/second) | <80 |
| Rapid (µm/second) | <120 |
| Progressivity (%) | 40% of straightness |
| Circular (%) | 50% of linearity |
| Connectivity | 11 |
| Velocity on the average path points | 7 |
| Number of images | 50 |

The analysis was done using Genstat Software. Analysis of variance (ANOVA) was used to test for differences between the treatments. Treatment means were separated using Fisher's protected t-test least significant difference at a significant level of P <0.05. The correlation of the body weight with semen volume, concentration and sperm motility was performed using SAS statistical software. The Pearson two-sided was used to determine the correlation between bodyweight and the variables (Snedecor & Cochran, 1980).

## 5.4 RESULTS

The results of Pearson's correlation coefficient (r) among the Kolbroek boars are outlined in Table 5.3. There was a positive correlation between bodyweight and semen volume of Kolbroek (r= 0.22) and Large White (r= 0.26). Additionally, a positive correlation existed between bodyweight and

sperm concentration for Large White (r= 0.37) but negatively correlated for Kolbroek sperm concentration (r= -0.66). Conversely, there was a negative correlation between bodyweight and sperm motility for both Kolbroek (r= -0.25) and Large White (r= -0.10) boars. A negative correlation was observed between semen volume and sperm concentration for Kolbroek (r= -0.73) and Large White (r= -0.30). There was a positive correlation between semen volume and sperm motility for Kolbroek (r= 0.08) and Large White (r= 0.91). Moreover, a positive correlation existed between sperm motility and sperm concentration for Large White (r= 0.47) but negatively correlated for Kolbroek (r= -0.25).

**Table 5.3** Pearson's correlation coefficient (r) among Kolbroek Large White for bodyweight and sperm characteristics

| Breed | Characteristics | Bodyweight (kg) | Semen volume (ml) | Sperm concentration ($x10^9$ sperm/ml) | Total motility (%) |
|---|---|---|---|---|---|
| Kolbroek | Bodyweight (kg) | 1.00 | | | |
| | Semen volume (ml) | 0.22 | 1.00 | | |
| | Sperm concentration ($x10^9$ sperm/ml) | -0.66 | -0.73 | 1.00 | |
| | Total motility (%) | -0.96 | 0.08 | 0.47 | 1.00 |
| Large White | Bodyweight (kg) | 1.00 | | | |
| | Semen volume (ml) | 0.26 | 1.00 | | |
| | Sperm concentration ($x10^9$ sperm/ml) | 0.37 | -0.30 | 1.00 | |
| | Total motility (%) | -0.10 | 0.91 | -0.25 | 1.00 |

The effect of different extenders and storage period (hours) on Kolbroek and Large White sperm motility rate is shown in Table 5.4. Kolbroek boar sperm motility rate declined during a 48 hour period, irrespective of the four semen extenders. Kolbroek boar semen maintained motility rate when diluted with Tris-based (89.5±5.1%) extender at 24 hours as compared to BTS (47.3±12.5 %), Kobidil$^{+}$ (48.6±16.9%) and Citrate (40.0±21.0 %) extenders. Furthermore, the highest sperm motility rate was achieved with Kolbroek semen diluted with Tris-based extender (74.1±8.5%) at 48 hours storage. Overall, semen diluted with BTS (28.6±36.1%), Kobidil$^{+}$ (15.6±5.8%) and Citrate (10.1±11.1%) extenders showed the lowest total sperm motility rate at 48 hours storage.

The percentage of sperm motility did not differ significantly among extenders throughout the storage period, although there was a significant reduction of sperm motility at 48 hour storage. Large White boar semen diluted with BTS (62.9±11.9%), Kobidil$^{+}$ (69.3±16.3%) and Tris-based extender (65.1±17.0%), showed a significantly higher sperm motility rate as compared to semen diluted with Citrate extender (27.6±17.1%).

**Table 5.4** Effect of different extenders and storage period (hours) on Kolbroek and Large White sperm motility rate (mean±SD)

| Breed | Treatments | 0 hours | 3 hours | 24 hours | 48 hours |
|---|---|---|---|---|---|
| Kolbroek | BTS | 91.2±5.5 [a] | 92.9±3.7 [a] | 47.3±12.5 [b] | 28.6±36.1 [a] |
| | Kobidil$^{+}$ | 92.7±4.6 [a] | 96.2±1.3 [a] | 48.6±16.9 [b] | 15.6±5.8 [b] |
| | Citrate | 94.5±5.4 [a] | 93.3±4.2 [a] | 40.0±21.0 [b] | 10.1±11.1 [b] |
| | Tris | 85.4±9.2 [a] | 93.5±7.6 [a] | 89.5±5.1 [a] | 74.1±8.5 [b] |
| Large White | BTS | 85.3±3.5 [a] | 81.4±11.0 [a] | 62.4±24.4 [a] | 62.9±11.9 [a] |
| | Kobidil$^{+}$ | 80.8±6.3 [a] | 77.3±9.6 [a] | 73.5±9.1 [a] | 69.3±16.3 [a] |
| | Citrate | 82.7±13.2 [a] | 76.89±11.7 [a] | 57.8±19.3 [a] | 27.6±19.1 [b] |
| | Tris | 83.0±8.5 [a] | 81.8±12.1 [a] | 71.3±22.1 [a] | 65.1±17.0 [a] |

BTS (Beltsville Thawing Solution). [ab] Different letters indicate significant differences (P<0.05).

## 5.5 DISCUSSION

There was a positive correlation between bodyweight and semen volume for Kolbroek (r= 0.22) and Large White (r= 0.26) and sperm concentration for Large White (r= 0.37) but negatively correlated for Kolbroek (r= -0.66). Conversely, a negative correlation existed between bodyweight and sperm motility for Kolbroek (r= -0.25) and Large White (r= -0.10). Moreover, a negative correlation was observed between semen volume and sperm concentration for both Kolbroek (r= -0.73) and Large White (r= -0.30). There was a positive correlation between semen volume and sperm motility for both Kolbroek (r= 0.08) and Large White (r= 0.91) boars. Furthermore, a positive correlation existed between sperm motility and sperm concentration for Large White (r= 0.47) but negatively correlated for Kolbroek (r= -0.25) boars. Kolbroek boar maintained sperm motility rate when diluted with Tris-based extender at 24 (89.5±5.1%) as compared to BTS (47.3±12.5 %), Kobidil$^{+}$ (48.6±16.9%) and Citrate (40.0±21.0 %) extenders. Large White boar semen diluted with BTS (62.9±11.9%), Kobidil$^{+}$ (69.3±16.3%) and Tris-based extender (65.1±17.0%), showed a significantly higher sperm motility rate as compared to semen diluted with Citrate extender (27.6±17.1%).

According to Peters *et al*. (2008), the relationship between semen volume, sperm motility and sperm concentration are important since they, to a large extent determine the fertility potential of semen. There was a positive correlation between bodyweight and semen volume of Kolbroek (r= 0.22) as well as total motility and sperm concentration (r= 0.47). Similarly, a positive correlation existed between sperm concentration and motility (r = 0.25) in chicken breeds (Peters *et al*., 2008). A negative correlation existed between semen volume and sperm concentration (r= -0.73). In contrast, a positive correlation between semen volume and sperm concentration was observed in chicken breeds (Schneider, 1992) and in Bengal bucks (Kabiraj *et al*., 2011). The negative correlation value obtained for semen volume and sperm concentration (r= -0.73) indicated that higher semen volume may not necessarily translate to higher sperm concentration. Furthermore, the correlation estimates obtained for semen volume and sperm motility (r= 0.08)

was expected because the more the semen volume, the more space is available for sperm to move easily.

Tris-based extender maintained a good motility (74.1±8.5%) up to 48 hours of storage for Kolbroek boars. BTS (62.9±11.9%), Kobidil (69.3±16.3%) and Kolbroek boars semen diluted with Tris based extender maintained sperm motility (74.1%). Minor decreases in motility of extended boar semen within the 24 hours of storage at 15 to 18 °C have previously been documented (Waberski *et al*., 1994; Weitze, 1991). This phenomenon was not observed in the present study, as there was a drastic decline in total and rapid sperm motility for Kolbroek boar sperm extended with BTS, Kobidil and Citrate. Kommisrud *et al*. (2002) reported a significant influence of boar on motility in semen stored for five days in BTS, suggesting variability exists among boars with regard to the ability to maintain sperm motility during storage. However, the findings of the present study were unexpected as the sperm total motility of Kolbroek boar semen extended with BTS was low (28.6%).

Large White sperm motility was gradually reduced during prolonged storage, a result which is in agreement with other investigations (Waberski *et al.* 1994). Huo *et al*. (2002) reported that Harbin White x Long White boar breeds were able to maintain sperm motility rate of 88.5% when extended with BTS after 48 hours. However, in the present study Large White boar sperm maintained a sperm motility of above 60% when extended with BTS, Kobidil and Tris-based extenders. The rapid motile sperm were low as compared to Kolbroek sperm at 24 and 48 hour storage, irrespective of extender. There might be variation in intrinsic properties of Kolbroek and Large White boar sperm membranes, possibly being of significance to sperm membrane functionality (Gadella *et al*., 1999) which might explain the differences.

Althouse *et al*. (1998) stated that a higher motility decreases sperm life through increased energy consumption. In contrast, Tris based-extender had sufficient nutrients to sustain and prolong the lifespan of Kolbroek boar sperm whilst BTS, Kobidil$^{+}$ and Tris-based extender also maintained high sperm motility rate for Large White boars. Tris-buffered extenders has also been

shown to be effective for preserving viability and fertilizing capability of rabbit spermatozoa stored at 15 °C (Roca *et al*., 2000). During storage, the boar spermatozoa undergo several changes including diminished motility, viability and alterations in membrane permeability (Kumaresan *et al*., 2008). This was evident in the present study as sperm motility declined over time. This may be due to boar sperm being sensitive to peroxidative damage due to the relative high content of polyunsaturated fatty acids in the phospholipids of the membrane (Cerolini *et al*., 2001) and the relative low antioxidant capacity of boar seminal plasma (Brezezinska-Slevbodzinska *et al*., 1995).

## 5.6 CONCLUSION

In conclusion, a positive correlation existed between bodyweight and semen volume of Kolbroek and Large White. There was a positive correlation between bodyweight and sperm concentration for Large White boars, but negatively correlated for Kolbroek boar sperm concentration. There was also a positive correlation between semen volume and sperm motility for both Kolbroek and Large White boars. Moreover, a positive correlation existed between sperm motility and sperm concentration for Large White but negatively correlated for Kolbroek sperm concentration. Kolbroek boar semen diluted with Tris-based extender managed to maintain a high sperm motility rate. However, sperm motility rate declined drastically for Kolbroek boar semen diluted with BTS, Kobidil$^{+}$ and Citrate extenders at 17°C. On the contrary, Large White boar semen diluted with BTS, Kobidil$^{+}$ and Tris-based extender maintained higher sperm motility rate at 17°C. Moreover, the SCA® has a great prospective for routine assessment of boar semen and in the development of new semen liquid storage techniques. However, future studies are required to determine the association between these SCA® measures and fertility.

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# CHAPTER 6

**Preliminary comparative evaluation of the different glycerol concentrations on boar semen characteristics prior to cryopreservation**

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## 6.1 Abstract

Glycerol is considered as an essential cryoprotectant for freezing boar sperm. Exposure of sperm to high glycerol concentration causes cell death. The aim of this study was to evaluate different glycerol concentrations (4 and 8%) on Kolbroek and Large White boar sperm motility rate prior to cryopreservation. Twelve ejaculates were collected separately from individual Kolbroek (n=4) and Large White (n=4) boars using the gloved-hand technique. Following semen collection, the semen was evaluated for macroscopic and microscopic characteristics. Semen was diluted with Beltsville Thawing Solution BTS extender. Extended sperm-rich fractions were slowly cooled to 17°C for three hours. Thereafter, the extended sperm suspension was centrifuged at 375 x *g* for 15 minutes at 17ºC. The supernatants were discarded and the pellets were further extended with egg yolk β-Lactose. After further cooling to 5°C and hold for two hours, the extended sperm were then diluted in LEY with glycerol (4 vs. 8%) concentration. Data was analyzed using GLM of SAS. Kolbroek boar total sperm motility rate was 89.1% for raw semen group with no significant differences compared to semen sample with 4% glycerol (69.0%).

However; a significant decrease of 69.0% sperm motility was recorded following the addition of 8% glycerol. Large White boar total sperm motility rate was 84.5% and decreased significantly following the addition of glycerol, irrespective of concentration level. In conclusion, breed differences were observed on boar sperm motility following the addition of glycerol. The addition of 4% glycerol resulted in better sperm motility for Kolbroek boars. It is recommended that 4% glycerol can be used during equilibration prior to cryopreserve Kolbroek boar semen.

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**Keywords:** Semen, CASA®, glycerol, equilibration time

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## 6.2 INTRODUCTION

The cryoprotective benefits of glycerol on spermatozoa were discovered by Polge *et al*. (1949) and are attributed mostly to its water-binding properties (reviewed by Salomon & Maxwell, 1995). Since then, the use of glycerol to preserve sperm during freezing is widespread (Hammerstedt et al., 1990; Bailey et al., 2000). Cryoprotectants aid in the freezing process by causing a hypertonic cellular environment and by modifying the sperm membrane to encourage water to leave the cell (Corcuera *et al*., 2007; Zeng *et al*., 2001). Primary factors causing sperm damage are phase transitions and separations in the sperm membrane, as well as intra- and extra- cellular ice crystal formation (Hammerstedt *et al*., 1990; Parks, 1997). However methods utilizing freezing mediums and cryoprotectants that account for these factors can limit damages due to cryopreservation, and have improved post-thaw sperm viability in the 60 years since the discovery of glycerol's importance (Fiser *et al*., 1993).

Fertilising integrity of frozen boar sperm is lower than that of fresh or liquid-stored semen (Ogbuewu *et al*., 2007; Umesiobi, 2008). The equilibration of extended semen at 5°C is an essential step in many protocols for cryopreservation of mammalian sperm (Sundararaman & Edwin, 2008).

Glycerol is currently the most common permeating cryoprotectant used in freezing livestock sperm (Holt, 2000). However, glycerol has to be used at low concentrations (under 4%) due to its potential toxicity for boar semen (Bhur *et al*., 2001). Exposure of cells to glycerol at either high concentrations or for prolonged periods causes cell death. Due to this toxic effect of glycerol, it is important to make certain the optimal concentration to give a cryoprotective effect while minimizing damage to the cell. The objective of this study was to find the suitable glycerol concentration (4 and 8%) prior to cryopreservation for Kolbroek and Large White boar using Computer-Aided Semen Analysis® (CASA®).

## 6.3 MATERIALS AND METHODS

The study was conducted at the Pig Research Unit of Agricultural Research Council (Germplasm Conservation & Reproductive Biotechnologies Unit), Irene, South Africa. The Agricultural Research Council-Irene campus is located at 25° 55' South; 28° 12' East. The institute is located in the Highveld region of South Africa and situated at an altitude of 1525m above sea level. Four indigenous Kolbroek and four exotic Large White boars were used for this study because of the scarcity challenge of finding Kolbroek boars. The boars were aged between two to four years of age. The study was done in summer season (February to March, 2011). The boars were in good health condition throughout the duration of the study. The diets were formulated to meet the nutritional requirements of the boars (NRC, 1998). Water was given *ad libitum* throughout the duration of the study.

Semen samples were collected from the experimental boars twice weekly from February to March. Twelve ejaculates were collected separately from four Kolbroek and four Large White boars with the gloved-hand technique in a 300 ml glass beaker. The filtered semen fraction were sealed with a gauze filter inside a pre-warmed (39ºC) insulated thermos flask. Experimental boars were cared for according to the guidelines for the Agricultural Research Council, Animal Production Institute ethics committee (Ref: APIEC10/01).

The 10 µl of raw semen was placed into 500 µl of swim up medium in a 15 ml tube (Falcon® 352099, USA). The tube was then kept in $CO_2$ incubator (Sanyo, Japan) adjusted to 39°C. Five µl of semen was placed on the warm glass slide (~76 x 26 x 1mm, Germany) and placed with a warmed cover slip (22 x 22 mm, Germany) over the microscope-warm plate (Omron) adjusted at 39°C. The sperm motility characteristics were evaluated by Computer Aided Sperm Analysis system (Sperm Class Analyser® 5.0, Microptic, Barcelona, Spain) at the magnification of 10x (Nikon, China). Before the track sequence was to be analysed, the trajectory of each sperm identified and recorded in each field was visually assessed to eliminate possible debris and to diminish the risk that unclear tracks were included in the analyses. The settings are shown on Table 6.1.

After assessing sperm motility rate, semen was extended with Beltsville Thawing Solution (BTS) and slowly cooled to 17ºC for 240 minutes. The extended sperm suspension was centrifuged at 375 x *g* for 15 minutes in a refrigerated centrifuge. The supernatants were discarded, and the pellets were re-extended with a β-Lactose–egg yolk extender. After further cooling to 5ºC and equilibrated for 120 minutes, the extended sperm were re-suspended with 94.5% β-Lactose, 4 or 8% glycerol.

**Table 6.1** The ARC Sperm Class Analyzer® settings used to analyse sperm motility and velocity characteristics

| Characteristics | Settings |
|---|---|
| Contrast | 169 |
| Brightness (MHz) | 470 |
| Image/second | 50 |
| Optic | Ph- |
| Chamber | Coverslide |
| Scale | 10X |
| Particle size ($\mu m^2$) | 10<70 |
| Slow (µm/second) | <40 |
| Medium (µm/second) | <80 |
| Rapid (µm/second) | <120 |
| Progressivity (%) | 40% of straightness |
| Circular (%) | 50% of linearity |
| Connectivity | 11 |
| Velocity on the average path points | 7 |
| Number of images | 50 |

The data were statistically analysed using Genstat Software. The experiment was designed as a completely randomised design with two treatments (Kolbroek and Large White boars). Analysis of variance (ANOVA) was used to test for differences between the treatments. The data were acceptably normal with homogeneous treatment variances. Treatment means were separated using Fisher's protected t-test least significant difference (LSD) at a significant level of $P<0.05$ (Snedecor & Cochran, 1980).

## 6.4 RESULTS

The effect of different glycerol concentrations on Kolbroek and Large White sperm motility and velocity rates is outlined on Table 6.2. Kolbroek boar sperm equilibrated in 4% glycerol yielded higher sperm motility rate

(81.4±6.3%) as compared to 8% (69.0±17.4%). However, no significant difference was observed for Large White boar semen equilibrated with 4 (35.5±15.4%) and 8% (37.1±16.0) glycerol concentration. Kolbroek boar semen in 4 and 8% glycerol maintained a higher proportion of sperm moving in faster (rapid) rate (61.2±17.3 and 51.6±14.0%, respectively), as compared to Large White boar semen group (35.5±15.4 and 35.4±15.4%, respectively). However, sperm with progressively motile percentage remained relatively low, irrespective of neither breed nor the glycerol concentration.

Glycerol equilibration resulted in highly significant increase of velocity on the curve line and velocity on the average path for Kolbroek boar sperm. Kolbroek boar semen with 4 and 8% glycerol showed a higher velocity on the curve line (179.6±34.8 and 182.8±22.6 µm/sec) as compared to Large White (132.9±25.9 and 135.9±27.6), irrespective of glycerol concentration. Kolbroek and Large White boar sperm showed an active type of movement (high VCL) but with a considerably reduced forward progression (lower velocity on the straight line and higher lateral head displacement), irrespective of glycerol concentration.

**Table 6.2** Effect of different glycerol concentrations on Kolbroek and Large White sperm motility and velocity rates (mean±SD)

| Characteristics | Kolbroek | | | Large White | | |
|---|---|---|---|---|---|---|
| | Raw | 4% Glycerol | 8% Glycerol | Raw | 4% Glycerol | 8% Glycerol |
| **TM (%)** | 89.1±11.9 [a] | 81.4±6.3 [a] | 69.0±17.4 [b] | 84.5±5.6 [a] | 66.3±9.6 [b] | 66.1±13.2 [b] |
| **RAP (%)** | 32.5±16.9 [c] | 61.2±17.3 [a] | 51.6±14.0 [ab] | 27.8±10.3 [c] | 35.5±15.4 [c] | 37.1±16.0 [bc] |
| **PM (%)** | 27.0±10.4 [a] | 23.4±7.4 [a] | 21.5±13.3 [a] | 29.1±11.9 [a] | 24.0±10.5 [a] | 22.2±5.8 [a] |
| **VCL (µm/sec)** | 111.6±16.2 [b] | 179.6±34.8 [a] | 182.8±22.6 [a] | 112.9±18.2 [b] | 132.9±25.9 [b] | 135.9±27.6 [b] |
| **VSL (µm/sec)** | 31.1±11.2 [a] | 29.3±8.0 [a] | 31.0±4.4 [a] | 30.6±7.8 [a] | 29.6±5.8 [a] | 33.3±7.0 [a] |
| **VAP (µm/sec)** | 67.3±20.1 [b] | 81.1±17.3 [a] | 82.0±9.2 [a] | 61.1±8.0 [b] | 64.3±10.0 [b] | 66.8±10.1 [b] |
| **LIN (%)** | 22.6±3.7 [a] | 16.5±3.3 [b] | 17.1±2.6 [b] | 27.0±4.4 [a] | 22.6±3.7 [ab] | 25.7±8.4 [a] |
| **STR (%)** | 46.3±5.9 [abc] | 36.4±7.1 [bc] | 38.0±5.2 [a] | 50.0±9.6 [a] | 46.3±5.9 [a] | 51.0±14.1 [a] |
| **ALH (µm/sec)** | 2.9±0.4 [c] | 4.7±0.9 [b] | 5.5±0.5 [a] | 3.3±0.5 [c] | 4.4±0.8 [b] | 4.4±0.7 [b] |
| **BCF (Hz)** | 19.6±2.2 [a] | 12.5±3.4 [bc] | 9.1±2.6 [d] | 15.3±2.3 [b] | 10.7±2.8 [cd] | 10.7±4.2 [cd] |

TM (total motility), RAP (Rapid), PM (progressive motility), VCL (velocity on the curve line), VSL (velocity on the straight line), VAP (velocity on the average path), LIN (linearity), STR (straightness), ALH (lateral head displacement), BCF (beat cross frequency). [abc] Different letters indicate significant differences within rows (P <0.05).

## 6.5 DISCUSSION

Raw total sperm motility rate was higher for Kolbroek (89.1±11.9%) and Large White (84.5±5.6 %) boars. Dilution of Kolbroek boar semen with 4% glycerol resulted in higher total sperm motility rate (81.4±6.3%) compared to 8% glycerol (69.0±17.4%). However, no significant difference was observed for total sperm motility rate of Large White boar semen equilibrated with 4 (66.3±9.6) and 8% (66.1±13.2) glycerol. Moreover, Kolbroek boar semen with 4 and 8% glycerol maintained a higher rapid sperm motility rate (61.2±17.3 and 51.6±14.0%, respectively), as compared to Large White boar semen (35.5±15.4 and 35.4±15.4%, respectively). Progressive sperm motility rate remained low (≤ 30%), irrespective of glycerol concentration and breed. A decrease in straightness and beat cross frequency was observed for Kolbroek boars, whilst velocity on the straight line remained constant. However, velocity characteristics for Large White boars remained constant, irrespective of glycerol concentration, except for beat cross frequency and lateral head displacement.

Sperm motility tended to declined following equilibration with glycerol. Similarly, sperm motility reduced following dilution with glycerol and slows cooling prior to freezing in Indian rhinoceroses (Stoops *et al*., 2010). According to Ponglowhapan & Chatdarong (2008), reducing the temperature to 4°C for 2 hours prior to freezing resulted in a decreased percentage of motile sperm (77.2 to 66.9%) in dogs. This demonstrates the toxicity of glycerol towards sperm prior to cryopreservation. The decline in sperm motility during equilibration may be due to the formation of peroxides from free radicals (Sundararaman & Edwin, 2008). Boar sperm seems to be sensitive to peroxidative damage due to the relative high content of polyunsaturated fatty acids in the phospholipids of the membrane (Cerolini *et al*., 2001) and the relative low antioxidant capacity of boar seminal plasma (Brezezinska-Slevbodzinska *et al*., 1995). Glycerol is a permeating agent that enters into the cell to replace the water that leaves during cooling, thus reducing the osmotic stress (Medeiros *et al*., 2002). However, glycerol may be toxic to the

cell and cause membrane instability and motility loss (Medeiros *et al*., 2002) which was evident in the present study.

Glycerol equilibration resulted in highly significant increase of velocity on the curve line and velocity on the average path, irrespective of the breed. In contrast, declines in velocity on the curve line and velocity on the average path were observed in Boer goats during equilibration. For cryopreservation of boar sperm, the quality of sperm motility in terms of speed is important in determining the post thaw survivability (Sundararaman & Edwin, 2008). Furthermore, the measurement of sperm motility is useful in predicting fertility (Budsworth *et al*., 1998). Microscopic techniques have limitations including subjectivity, variability, the small number of sperm analysed and poor correlation with fertilizing potential (Rijsselaere *et al*., 2005). Subjective visual evaluation of motility is also prone to human error and biasness. Hence, the computer-assisted sperm analysis (CASA) was initiated to reduce subjective bias on the motility assessment and to discriminate a series of motility patterns of boar semen (Tretipskul *et al*., 2010).

## 6.6 CONCLUSION

Semen from Kolbroek tolerated glycerol concentration level better than Large White boar. The inclusion of 4% glycerol resulted in high sperm motility in Kolbroek boars. Furthermore, Kolbroek boar semen diluted and hold for two hours in 4 and 8% glycerol maintained a higher percentage of rapid sperm motility. Large White boar semen resulted in lower sperm motility proportions, irrespective of the glycerol concentration. This study also revealed that the exposure of sperm to the cryoprotectant during equilibration or holding prior to cryopreservation is detrimental sperm in both breeds especially in Large White boar.

## 6.7 ACKNOWLEDGEMENTS

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# CHAPTER 7

**High conception rate following artificial insemination by frozen-thawed Kolbroek and Large White boar semen**

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## 7.1 ABSTRACT

Frozen-thawed boar sperm holds the potential to have an impact on the future of the swine industry. The aim of the study was to determine the pregnancy rate following artificial insemination by frozen-thawed semen. Twelve ejaculates were collected separately from individual Kolbroek (n=4) and Large White (n=4) boars using the gloved-hand technique. Following semen collection, semen was evaluated for macroscopic and microscopic characteristics. Semen was diluted with Beltsville Thawing Solution (BTS) extender and cooled to 17°C for 240 minutes. Thereafter, the extended semen was centrifuged at 375 x *g* for 15 minutes at 17ºC. The sperm pellet was extended with β-Lactose egg yolk. After further cooling to 5°C and equilibrated for 120 minutes, the extended sperm were then suspended with 94.5% β-Lactose, 4% glycerol and 1.5% Equex Es Paste. The semen straws were exposed to liquid nitrogen vapour at four cm above liquid nitrogen for eight minutes then plunged directly into liquid nitrogen. Semen straws were thawed at 37°C for 20 seconds. Thirty three sows, aged two to four years, were bred at 12 and 24 hours after first observed in standing oestrus using semen from Kolbroek and Large White boars, diluted in BTS short-term

extender and cryopreserved semen. Cryopreservation affected post thawed sperm motility rate for Kolbroek (30.2±5.2%) and Large White (24.0%) boar. Higher pregnancy rate was recorded in Kolbroek (80.0%) and Large White (81.3%) sows inseminated with diluted raw boar semen compared to lower pregnancy rate in sows inseminated with Kolbroek (50%) and Large White (50%) frozen-thawed semen. In conclusion, cryopreservation significantly reduced sperm motility rate pregnancy rate as compared to the diluted raw boar semen, irrespective of the breed. Moreover, frozen-thawed resulted in lower pregnancy rate as compared to diluted boar, irrespective of the breed.

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Keywords: Sperm, Large White, Kolbroek, Cryopreservation, CASA®

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## 7.2 INTRODUCTION

Artificial insemination (AI) is the oldest and currently the most common used assisted reproductive technology and an important tool in animal production (Vishwanath, 2003). High reproductive performance is a key factor for optimal economic success in swine production (Gröhn & Rajala-Schultz, 2000). Furthermore, swine AI involves almost exclusively the use of liquid semen collected within the previous 3–7 days, although there is evidence to indicate that optimal fertility requires AI within 48 hours after collection and extension in both short- and long-term extenders (Waberski *et al*., 1994). The application of an effective sperm cryopreservation is envisaged to provide a remedy to the current drain of genetic resources and would ensure long-term availability of male gamete (Geerlings *et al*., 2002).

Cryopreservation is defined as the freezing of tissues or cells to preserve them indefinitely for future use (Dinnyes *et al*., 2007). Even though cryopreserved boar semen has been available since 1975, a major breakthrough in commercial application has not yet occurred (Groβfeld *et al*., 2008) to date. Boar spermatozoa are extremely sensitive to cold shock, possibly because of the low cholesterol/phospholipid ratio of their membranes (Johnson *et al*. 2000). Therefore, the success of fertilization with frozen-

thawed semen differs significantly between boar and other species like bull (Holt, 2000a; Holt, 2000b; Johnson *et al*., 2000). Previously, approximately 40–50% of the sperm did not survive cryopreservation (Watson, 2000). These low post thawed results were believed to be a result of sperm cryo-injury (Watson, 2000) including, but not limited to, a capacitation-like or premature aging effect (Bravo *et al*., 2005; Ortega-Ferrusola *et al*., 2008), associated with an increase in DNA fragmentation (Fraser & Strzezek, 2007). Fertility following AI using frozen-thawed semen has yielded pregnancy rates ≥60% (Eriksson *et al*., 2002; Hofmo & Grevel, 2000; Roca *et al*., 2006a). The aim of the study was to determine the pregnancy rate following artificial insemination by frozen-thawed semen.

## 7.3 MATERIALS AND METHODS

The study was conducted at the Pig Research Unit of Agricultural Research Council (Germplasm Conservation & Reproductive Biotechnologies Unit), Irene, South Africa. The Agricultural Research Council-Irene campus is located at 25° 55’ South; 28° 12’ East. The institute is located in the Highveld region of South Africa and situated at an altitude of 1525m above sea level. Four indigenous Kolbroek and four exotic Large White boars were used for this study because of the scarcity challenge of finding Kolbroek boars. The boars were aged between two to four years of age. The study was done in summer season (February to March, 2011). The boars were in good health condition throughout the duration of the study. The diets were formulated to meet the nutritional requirements of the boars (NRC, 1998). Water was given *ad libitum* throughout the duration of the study.

Twelve ejaculates were collected separately from four Kolbroek and four Large White boars with the gloved-hand technique in a 300 ml glass beaker. The filtered semen fraction were sealed with a gauze filter inside a pre-warmed (39ºC) insulated thermos flask. Experimental boars were cared for according to the guidelines for the Agricultural Research Council, Animal Production Institute ethics committee (Ref: APIEC10/01).

Semen samples were collected from the experimental boars twice weekly throughout the three weeks of research experimentation. Twelve ejaculates were collected separately from the experimental boars using the gloved-hand technique and semen deposited in a 300 mL glass beaker. Sperm motility was evaluated using sperm Class Analyzer®. After assessing sperm motility, semen was extended with Beltsville Thawing Solution (BTS) and slowly cooled to 17ºC for 240 minutes. The extended sperm suspension was centrifuged at 375 x *g* for 15 minutes in a refrigerated centrifuge. The supernatants were discarded, and the pellets were re-extended with a β-Lactose–egg yolk extender. After further cooling to 5ºC and equilibrated for 120 minutes, the extended sperm were re-suspended with 94.5% β-Lactose, 4% glycerol and 1.5% Equex Es Paste. The extended and cooled sperm were packed into 0.5-mL polyvinyl chloride and placed in contact with liquid nitrogen vapour about four cm above the liquid nitrogen level for eight minutes in an expandable polystyrene box then plunged into the nitrogen tank (-196ºC). Straws were thawed in a circulating water bath at 37ºC for 20 seconds. Straws semen samples were thawed at 37ºC in BTS (1:2) and evaluation of sperm motility was done using SCA®.

The 10 µl of raw semen were placed into 500 µl of BO wash medium in 15 ml tube (Falcon® 352099, USA). The tube was then kept in $CO_2$ incubator (Sanyo, Japan) adjusted to 39°C. Five µl of semen was placed on the warm glass slide (~76 x 26 x 1mm, Germany) and placed with a warmed cover slip (22 x 22 mm, Germany) over the microscope-warm plate (Omron) adjusted at 39°C. The sperm motility rates were evaluated by Computer Aided Sperm Analysis system (Sperm Class Analyzer® [SCA] 5.0, Microptic, Barcelona, Spain) at the magnification of 10x (Nikon, China). The kinematic values recorded for each sperm included, in addition to the overall percentage of motile sperm, the velocity of movement, the width of the sperm head's trajectory, and the frequency of the change in direction of the sperm head (Table 7.1).

**Table 7.1** The ARC Sperm Class Analyzer® settings used to analyse sperm motility and velocity characteristics

| Characteristics | Settings |
|---|---|
| Contrast | 169 |
| Brightness | 470 |
| Image/second | 50 |
| Optic | Ph- |
| Chamber | Cover slide |
| Scale | 10X |
| Particle size ($\mu m^2$) | 10<70 |
| Slow (µm/second) | <40 |
| Medium (µm/second) | <80 |
| Rapid (µm/second) | <120 |
| Progressivity (%) | 40 % of straightness |
| Circular (%) | 50 % of linearity |
| Connectivity | 11 |
| Velocity on the average path points | 7 |
| Number of images | 50 |

Thirty three sows, aged two to four years, were bred at 12 and 24 hours after first observed in standing oestrus using semen from Kolbroek and Large White boars, diluted in BTS short-term extender and cryopreserved semen. The semen was used within three hours after collection. The proportion of experimental sows that expressed oestrus and were mated, were observed for pregnancy using Draminski ultrasonography (Figure 7.1).

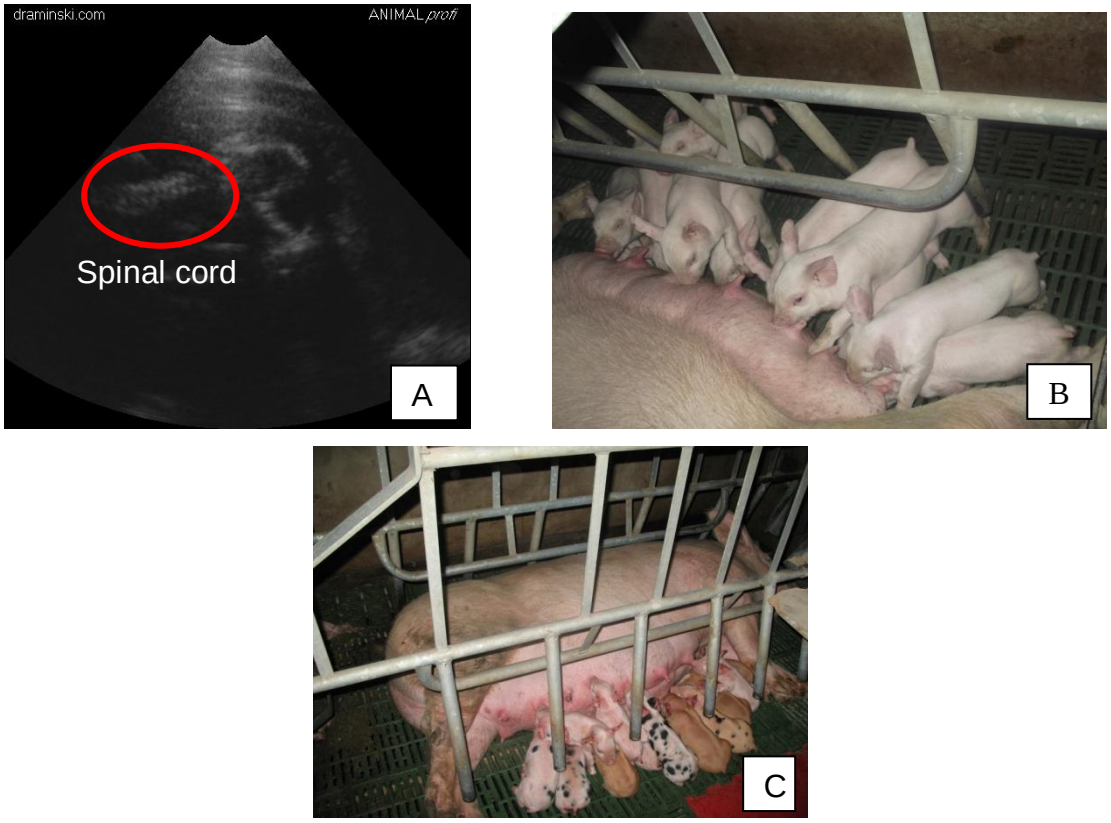


**Figure 7.1** Scanned image of a pregnant Large White sow using Draminski ultrasonography (A); an image of Large White piglets (B) and Kolbroek x Large White piglets (C).

The data were statistically analysed using Genstat Software. Analysis of variance (ANOVA) was used to test for differences between the treatments. Treatment means were separated using Fisher's protected t-test least significant difference at a significant level of $P < 0.05$.

## 7.4 RESULTS

As shown in Table 7.2, post thawed semen samples from both breeds showed a significant ($P < 0.05$) decrease in total sperm motility rate. The total sperm motility of Kolbroek (94.4±1.6%) and Large White (85.0±8.9%) boar significantly declined for both Kolbroek (30.2±5.2%) and Large White (24.0±11.9%) boars.

**Table 7.2** Sperm motility rates of raw and frozen-thawed Kolbroek and Large White boars (mean±SD)

| Breed | Treatments | TM (%) | RAP (%) | PM (%) | NPM (%) | SLO (%) | MED (%) |
|---|---|---|---|---|---|---|---|
| Kolbroek | Raw | 94.4±1.6[a] | 25.4 ±16.9[a] | 39.0±17.8 [a] | 55.4±16.8 [a] | 27.5±8.1 [a] | 41.5±26.3 [a] |
| | Frozen-thawed | 30.2±5.2[b] | 12.6 ±11.3[a] | 18.9±2.8 [a] | 11.3±6.8 [b] | 5.4±9.4 [a] | 12.2±11.5 [a] |
| Large White | Raw | 85.0±8.9[a] | 35.7 ±16.3[a] | 26.7[a]±12.0 | 58.3±20.1 [a] | 32.6±26.5 [a] | 16.7±10.0 [a] |
| | Frozen-thawed | 24.0±11.9[b] | 9.0 ±3.6[a] | 15.8[a]±7.9 | 8.2±5.8 [b] | 7.4±4.6 [a] | 7.5±6.3 [a] |

TM (Total motility); RAP (rapid motility); PM (progressive motility); NPM (non progressive motility); SLO (slow motility); MED (medium motility). [abc] Different letters indicate significant differences within rows at $P > 0.05$.

**Table 7.3** Sperm velocity rates for raw and frozen-thawed Kolbroek and Large White boar semen (mean±SD)

| Breed | Treatments | VCL (µm/sec) | VSL (µm/sec) | VAP (µm/sec) | LIN (%) | STR (%) | WOB (%) |
|---|---|---|---|---|---|---|---|
| **Kolbroek** | **Raw** | 110.5±17.8 [a] | 31.5±3.2 [a] | 63.1±12.4 [a] | 28.9±5.1 [a] | 51.6±14.1[b] | 57.0±6.4 [a] |
| | **Frozen-thawed** | 120.0±66.7 [a] | 67.8±36.9 [a] | 60.2±15.9 [a] | 35.5±10.4 [a] | 58.2±17.1[b] | 34.4±24.3 [a] |
| **Large** | **Raw** | 123.6±36.2 [a] | 27.6±6.8 [a] | 57.0±33.5 [a] | 22.5±2.5 [a] | 46.4±10.0[b] | 49.2±6.2 [a] |
| **White** | **Frozen-thawed** | 106.2±20.4 [a] | 53.3±25.2 [a] | 62.3±21.8 [a] | 49.1±15.9 [a] | 83.1±10.3[a] | 58.2±13.1 [a] |

VCL (velocity on the curve line); VSL (velocity on the straight line); VAP (velocity on the average path); LIN (linearity); STR (straightness) and WOB (wobble).[ab] Different letters indicate significant differences within rows (*P* >0.05).

The results of both breed boars sperm velocity are outlined on Table 7.3. Raw and frozen-thawed sperm showed an active type of movement characterized by curvilinear velocity ranging from 106.2 to 123.6 µm/sec; however, raw boar sperm show a considerably reduced forward progression (straight-line velocity: 31.5±3.2 and 27.6±6.8 µm/sec) as opposed to the frozen-thawed sperm (straight-line velocity: 60.2±17.8 and 62.3±21.8 µm/sec) for Kolbroek and Large White boars, respectively. No significant difference was observed for linearity and wobble regardless of breed and treatment, except straightness.

The effect of breed and pregnancy rate following artificial insemination by frozen-thawed boar semen is shown in Table 8.4. The fertility rate of diluted and frozen-thawed boar sperm following artificial insemination is outlined on Table 7.3. Higher pregnancy rate was attained for Kolbroek (100.0%) and Large White (81.3%) sows for diluted boar semen. Lower pregnancy rate was attained for sows inseminated with Kolbroek (50%) and Large White (50%) frozen-thawed semen.

**Table 7.4** Effect of breed and pregnancy rate following artificial insemination by frozen-thawed boar semen

| **Breed** | **Treatment** | **Number of sows (n=33)** | **Pregnancy rate (%)** |
|---|---|---|---|
| Kolbroek | Diluted semen | 5 | 5/5 (100%) |
| | Frozen-thawed | 6 | 3/6 (50%) |
| Large White | Diluted semen | 16 | 13/16 (81%) |
| | Frozen-thawed | 6 | 3/6 (50%) |

**DISCUSSION**

Raw sperm total motility rate was 94.4±1.6 and 85.0±8.9% for Kolbroek and Large White boars, respectively. Freezing and thawing process caused a dramatic deterioration in sperm total motility rates for Kolbroek (30.2±5.2%) and Large White (24.0±11.9%). Moreover, higher pregnancy rate was achieved for Kolbroek (80.0%) and Large White (81.3%) sows for diluted boar semen. However, frozen-thawed

resulted in lower pregnancy rate for Kolbroek (50%) and Large White (50%) as compared to diluted boar semen.

The total motility rate was 94.4±1.6 and 85.0±8.9% for Kolbroek and Large White boars, respectively. Furthermore, no significant difference (*P* >0.05) was observed for rapid sperm motility, progressive motility, slow and medium sperm motility rate, irrespective of breed. However, there was a significant difference between raw and frozen-thawed sperm for non-progressive motility, irrespective of the breed. Post-thaw motility rate for Kolbroek (30.2%) and Large White (24.0%) were lower than the 50% motility rate reported by (Eriksson *et al*., 2002; Roca *et al*., 2003). Previously, low post thaw sperm motility rates were observed for Norwegian Landrace (36.3%) and for Duroc (19.6%) boars (Hofmo & Grevle, 1999). These findings are in agreement with the present study. In contrast, Egerszegi *et al*. (2008) reported higher post thaw sperm motility (56.2±6.18%) in Hungarian indigenous Mangalica boars.

Moreover, of significant interest is the report by Mapeka *et al*. (2009) which suggested a post thaw total motility of 61.0±12.1% for indigenous Kolbroek boars. However, subjective analysis was used for this finding which is prone to human error and biasness. Microscopic techniques have limitations including subjectivity, variability, the small number of sperm cell analysed and poor correlation with fertilizing potential (Rijsselaere *et al*., 2005). Hence, the computer-assisted sperm analysis® (CASA®) was used to reduce subjective biasness on the motility assessment and to discriminate a series of motility patterns of boar semen (Tretipskul *et al*., 2010).

Schmidt & Kamp. (2004) defined hyperactivated boar sperm with high curvilinear velocity (≥97 µm/sec) and amplitude of lateral head displacement (≥3.5 µm/sec) and low Linearity (≤35%). In contrast, higher sperm Linearity percentages were observed for Kolbroek and Large White boars post thaw semen (35.5±10.4 and 49.1±15.9%). Compared with other animal species, the sperm Linearity of Kolbroek and Large White boars are rather low. In dog semen, Iguer-ouada & Verstegen (2001) found a Linearity of 55 ± 10%. An increase in Linearity was observed when sperm motility was assessed in raw Large White semen (22.5±2.5%) and after freezing and

thawing (49.1±15.9%). Similarly, such changes in sperm kinematics have been described previously (Pena *et al*., 2003). There was an increase of VSL in Kolbroek raw semen (31.5±3.2 µm/sec) and frozen-thawed semen (67.8±36.9 µm/sec) as well as for Large White raw boar semen (27.6±6.8 µm/sec) and frozen-thawed semen (53.3±25.2 µm/sec). In contrast, it was reported that VSL and VAP do not increase after freezing and thawing (Pena *et al*., 2003).

In the present study, the boar sperm motility was significantly decreased after the freezing and thawing procedure and an approximate reduction of 60% was observed in sperm motility. This is because the process of cryopreservation, including freezing and thawing, induces structural and/or biochemical damage in boar sperm, resulting in a drastic reduction of the percentages of motile sperms (Cremades *et al*., 2005). The reason may also be due partly to the lipid structure within the plasma membrane of the boar s that is very sensitive to the change in temperature (Johnson *et al*., 2000). The susceptibility to cryo-damage of the boar sperm is related to the differences of individual boars and the breed of boar (Thurston *et al*., 2002; Holt *et al*., 2005).

In the present study, higher pregnancy rate was achieved for sows inseminated with Kolbroek (100.0%) and Large White (81.3%) boar semen. However, a pregnancy rate of 50% was obtained for sows inseminated with both Kolbroek and Large White boar semen. The doses with ≥50% motility have been used to achieve the highest reported pregnancy rate in gilts at day 30 (65%), while the highest reported pregnancy rate in sows at day 30 was 79% (Eriksson *et al*., 2002; Eriksson & Rodriguez-Martinez, 2000). These results may be due to the longer survival of shortly preserved spermatozoa in sow oviduct (up to 20 hours) compared to frozen-thawed sperm (Eriksson *et al*., 2002). Frozen-thawed sperm has a much shorter lifespan, compared to liquid semen, due to the damage that occurs during the cooling and freezing phases of cryopreservation and the capacitated-like state that results (Bailey *et al*., 2008; Waberski *et al*., 1994). However, the main deterrent for utilizing frozen-thawed sperm for breeding purposes is the reduced farrowing rates < 70% and reduced litter sizes (Roca *et al*., 2006b; Watson, 2000).

## 7.6 CONCLUSION

Raw total sperm motility declined drastically following freezing and thawing, with very low post-thaw viability for both boar breeds. Furthermore, frozen-thawed resulted in lower pregnancy rate as compared to diluted boar semen, irrespective of the breed. However, low motility rate from frozen thawed semen resulted in higher conception rate. Further studies are required to improve the existing freezing and thawing protocol for boar semen. Moreover, alternative technologies such as deep uterine insemination may allow for further perfection of breeding techniques with frozen-thawed boar semen.

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# CHAPTER 8

## 8.1 GENERAL CONCLUSIONS

The bodyweight of Kolbroek and Large White boar was positively correlated with ejaculated semen volume. However, macroscopic and microscopic sperm characteristics of Kolbroek were similar compared to Large White boar. Kolbroek boar sperm concentration and motility rate was negatively correlated to bodyweight compared to only Large White sperm concentration. This is the first study that provided more information on sperm motility characteristics using Sperm Class Analyser® and correlations that exist between bodyweight and sperm characteristics of both Kolbroek and Large White boars.

Temperature and storage period did not affect the sperm motility rate of Large White; however, sperm motility rate of Kolbroek boar was affected by temperature and storage period. Kolbroek and Large White boar sperm stored at 17°C for 24 hours maintained the acceptable sperm motility rate. Thus, Kolbroek and Large White boar semen may be transported overnight to any South African Developing Countries when stored at 17°C within 24 hours for artificial insemination. The SCA® showed that it has a great potential for routine evaluation of boar semen and in the development of new semen liquid storage methods.

Kolbroek boar semen diluted with Tris-based extender maintained a high sperm motility rate. However, sperm motility rate declined drastically for Kolbroek boar semen diluted with BTS, Kobidil$^{+}$ and Citrate extenders at 17°C. On the contrary, Large White boar semen diluted with BTS, Kobidil$^{+}$ and Tris-based extender maintained higher sperm motility rate as compared to Citrate extender at 17°C.

The 4% glycerol resulted in high sperm motility in Kolbroek boars. Furthermore, Kolbroek boar semen diluted and hold for 2 hours in 4 and 8% glycerol maintained a higher percentage of rapid sperm motility rate at 5°C. Large White boar semen resulted in lower sperm motility proportions, irrespective of the glycerol concentration. This study also revealed that the exposure of sperm to the cryoprotectant during equilibration or holding prior to cryopreservation is detrimental sperm in both breeds especially in Large White boar.

Raw total sperm motility declined drastically following freezing and thawing, with very low post-thaw survival rate for both boar breeds. Furthermore, frozen thawed resulted in lower pregnancy rate as compared to diluted raw semen. This study showed that cryopreservation significantly reduced sperm motility regardless of breed however the pregnancy rate from frozen-thawed semen was high (50%), regardless of the breed.

## 8.2 RECOMMENDATIONS

- It is recommended that further studies should be conducted with more number of boars to validate the sperm motility characteristics information following artificial insemination.
- Improvement of the existing protocols for boar semen cryopreservation would be in the best interests of the pig industry. Furthermore, this would be essential to improve fertility rates in South African pig breeds.
- Furthermore, future studies are required to determine suitable short term extender for indigenous Kolbroek boars for a period of more than 72 hours.
- Moreover, alternative technologies such as deep uterine insemination may allow for further perfection of breeding techniques with frozen-thawed boar semen.