IMPROVEMENTS IN THE VIABILITY AND FERTILIZING INTEGRITY OF BOAR SPERMATOZOA USING THE “UMQOMBOTHI” *SORGHUM BICOLOUR* SEMEN EXTENDERS

TEELE PITSO

MAY 2009

IMPROVEMENTS IN THE VIABILITY AND FERTILIZING INTEGRITY OF BOAR SPERMATOZOA USING THE "UMQOMBOTHI" *SORGHUM BICOLOUR* SEMEN EXTENDERS

by

Teele Pitso

Dissertation submitted in partial fulfilment of the requirements

for

the degree

MAGISTER TECHNOLOGIAE

AGRICULTURE: ANIMAL PRODUCTION

in the

Faculty of Health and Environmental Science

School of Agriculture and Environmental Science

at the

Central University of Technology, Free State

Supervisor: Prof. DO Umesiobi (PhD, MA HES)

MAY 2009

ACKNOWLEDGEMENT OF FINANCIAL SUPPORT

I wish to express my sincere gratitude and appreciation to the Central University of Technology, Free State and National Research Foundation (NRF) for their financial contribution to the successful completion of this study.

DEDICATION

I dedicate this work to the extended and caring Teele family, my father Ramakoloi Teele, my mothers Mapheello, Mmatebello, Mmakamohelo, Mmatumo and Mmapaballo, my bothers Lefu, Thabo, Katleho, Tshepo, Potso, Lebohang, Tumelo, Koena, Tshepang, my sisters Matshepiso, Dipolelo, Palesa, Motlalepula, Motshidisi, Lerato, Malefa, Naledi Karabo and Toka, my nieces and nephews as well as my loving girlfriend Dilahloane Mojakhomo who has offered tremendous support through out this study.

DECLARATION

I, Pitso Teele, identity number [redacted] student number 207072345, declare that this dissertation: Improvements in the viability and fertilizing integrity of boar spermatozoa using the “umqombothi” (S*orghum bicolour)* semen extenders 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.

_____ _____

TEELE PITSO DATE

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor Dr DO Umesiobi of the Central University of Technology, Free State, for the vital role he has played in this project from the beginning to the end; Dr Umesiobi, your assistance, leadership and financial support are highly appreciated. I would also like to extend my thanks to my co-supervisor Dr TL Nedembale for his valuable support, patience and technical guidance throughout this project.

I would also like to express my gratitude to the National Research Foundation (NRF) for their financial support from day one up to the end of the project, as well as to the Agricultural Research Council (ARC) at Irene, for allowing me to utilise their laboratory at the Germplasm Conservation Reproduction Biotechnologies (GCRB) as well as the experimental animals from the animal nutrition unit.

My gratitude is also extended to the members of my family, my beloved mother Mmapheello, my father Ntate Teele, my brother Tshepo who encouraged me to initiate this project, my friend Gagi for his loyalty and trust in my study, my brothers, my sisters and friends for their prayers and moral support they offered.

Above all, I thank Almighty God for giving me strength and courage during the difficult times when I sweated night and day to put this project together and for making it possible for accomplishment of this study.

TABLE OF CONTENTS

Chapter 1

Orientation of study

Chapter 2

Literature review

Chapter 3

Research design and methodology

Chapter 4

Results and discussions

Chapter 5
Conclusions and recommendations

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ACRONYMS AND ABBREVIATIONS

Abbreviation	Description
%	Percent
°C	Degrees Celsius
AI	Artificial Insemination
ANOVA	Analysis of Variance
AO	Acridine Orange
ARC	Agricultural Research Council
Bmr	Brown Midrib
BSA	Bovine Serum Albumin
BTS	Beltsville Thawing Solution
CRD	Completely Randomised Design
CUE	Cornell University Extender
CWS	Coconut Water Solution
DAR	Damaged Apical Ridge
DE/kg	Digestible Energy per Kilogram
DNA	Deoxyribonucleic Acid
GCRB	Germplasm Conservation Reproduction Biotechnologies
h	Hour
HSP	Heat Shock Protein
ITS	Insulin-Transferrin-Selenium
Kg	Kilogram
LAR	Loose Apical Ridge
LS	Least Square
MAR	Missing Apical Ridge
ml	Millilitres

NRR	Non-Return Rate
NS	Not Significant
ROS	Reactive Oxygen Species
RP	*Raphia hookeri*
RPS	Palmwine plus 'Nche'
SAS	Statistical Analysis System
SCSA	Sperm Chromatin Structure Assay
SE	Standard Error
SP	Seminal Plasma
UMQ	Semen Extended in "Umqombothi"
UNX	Unextended Semen
vs	Versus

ABSTRACT

Key words: *Semen fertility, semen extender, Sorghum bicolour, artificial insemination, Large White Pigs*.

The objective of this study was to evaluate the viability of semen extended in "Umqombothi" (UMQ) and compare with Beltsville Thawing Solution (BTS) and unextended semen (UNX). Twelve large white boars and twelve large white sows were used in this experiment. The following sperm characteristics were measured; sperm motility percentage, live sperm, sperm concentration, abnormal sperm percentage and semen pH of (UNX), (UMQ) and (BTS) and compared, fertility parameters namely; non-return rate percentage, farrowing rate, total piglets and live piglets were also measured and compared.

The results from the study showed a significant difference *($p<0.05$)* in sperm motility between (UNX), (UMQ) and (BTS) whereby (UMQ) had the highest percentage of motile sperm which was followed by (BTS) and (UNX) having the lowest percentage of motile sperm, however the results also showed that sperm motility and live sperm percentage of semen stored at 4°C differed significantly *($p<0.05$)* from sperm motility and live sperm percentage of semen stored at 25°C whereby sperm motility and live sperm percentage of semen stored at 25°C were higher than sperm motility and live sperm percentage of semen stored at 4°C. Nevertheless no significant difference in sperm concentration and semen pH was found when semen stored at 4°C and 25°C were compared. However were time of semen collection of 9:00 and 15:00 were compared no significant differences in sperm motility percentage, live sperm percentage, sperm concentration, abnormal sperm percentage and semen pH were observed.

The study also revealed a significant difference *(p<0.05)* in non-return rate, farrowing rate, total piglets and live piglets between semen stored at 25°C and 4°C of which the results explain that semen stored at 25°C had a higher percentage of non-return rate , farrowing rate, total piglets and live piglets, however, Under (UNX) collected at 9:00 and 15:00 that there was no significant difference in no-return rate percentage, farrowing rate, total piglets and live piglets was observed when two times of semen collections were compared. Under (UMQ) collected at 9:00 and 15:00 there was also no significant difference in non-return rate percentage, farrowing rate, total piglets and live piglets observed when two times of semen collections were compared. Under (BTS) collected at 9:00 and 15:00 there was also no significant difference in non-return rate percentage, farrowing rate, total piglets and live piglets observed when two times of semen collections were compared. Nevertheless were semen extenders were compared (UNX) collected at 9:00 and 15:00 differed significantly *(p<0.05)* from (UMQ) and (BTS) collected at 9:00 and 15:00 whereby (UNX) had the lowest percentage of non-return rate, farrowing rate, total piglets and live piglets.

Chapter 1

Orientation

1.1 INTRODUCTION

Pig meat represents about 40% of all red meat consumed worldwide and continues to be an important part of the human diet throughout the world (Chung *et al*., 1998). In the past 10 years, pork production has increased from 73 to 94 million metric tons, according to FAO (2002). It is projected that the demand for pork will increase to 125 million metric tons by 2020 (Delgado *et al*., 1999), with most of the increase projected for developing countries. The improvement in efficiency of pork production, especially in recent years, is the result of implementation of several new biotechnological techniques and production practices. Major research advances have been made in genetics, nutrition, and disease and parasite control. The key to widespread application of artificial insemination (AI) worldwide is the ability to store semen extended in buffers for up to a week near room temperature. There is no doubt that improved extender composition has fueled the use of AI. However, one is unable to document the specific ingredients of 5–10 day extenders because most of the chemical formulas of extenders currently being marketed are proprietary (Delgado *et al*., 1999). Even with the advent of longer-term storage of semen, the majority of producers are inseminating sows on the first, second or third day following collection.

The use of semen extenders in the pig industry has been in existence for quite a number of years. However, researchers and farmers are still striving for the most appropriate measures of preserving semen. Research is still faced with problems of improving the viability and fertilizing integrity of boar spermatozoa, mostly because of the limited lifespan of the boar spermatozoa (Umesiobi, 2008a). The storage tolerance of boar spermatozoa depends predominantly on the choice of the extender (Levis, 2000; Umesiobi *et al*., 2002). Improvements in boar semen

require reliable and affordable semen extenders (Waberski *et al.,* 1994b), as well as conducive storage temperatures. The inability of stored boar semen to be extended in liquid form for several days without a significant reduction in fertility severely limits the optimum utilisation of boar semen for artificial insemination (Umesiobi, 2000a, b; Umesiobi *et al*., 2000). Thus, increasing interest in longer preservation of diluted sperm raises questions in the field concerning the choice of the extender. There is extensive use of artificial insemination in the pig industry; extended liquid boar semen may be used for insemination for up to 5 days after collection (Kuster & Althouse, 1999). Every extender provides the sperm cells with components which ensure a source of energy, proper pH and osmotic pressure. It also prevents thermal shock and inhibits bacterial growth. Other substances such as Heat Shock Protein (HSP) are being sought to improve the preserving properties of the extender (Curry, 2000). It was discovered, however, that some of the extender components may cause an increase in acrosome damages thus reducing semen fertility (Kuster & Althouse, 1999). Umesiobi (2004) evaluated the functional integrity of boar spermatozoa and sow fertility using Nigerian local semen extender Raphia (*Raphia hookeri*) Palmwine plus Nche (*Saccoglottis gabonensis*) urban extender in comparison with Cornel University extender. The author concluded that sperm motility, live sperm percent and sperm concentration were highest in semen extended in raphia palmwine + *S. gabonensis* extender followed by Cornel University extender. This finding could be an indication that “Umqombothi” extenders might also give positive results from the proposed study.

The problem with most of conventional extenders is that they are mostly developed for bovines rather than for pigs and they are also designed to accommodate semen life-span of more than 16 hours. This will be a disadvantage for pigs, as they yield low artificial insemination doses per ejaculate (Chung *et al*., 1998), mostly due to low cell survival, and subsequently resulting in both low fecundity rates and low survival rates (Gillmore *et al*., 1998). The principal advantage of using unfrozen, liquid semen is that fertility is maintained

even with low numbers of spermatozoa in the inseminate (Chung *et al*., 1998) but fertility of liquid semen is lost during extended periods at ambient temperatures. Moreover, conventional semen extenders were found to be expensive and in some cases unreliable. What can make this unreliable might be that the survival of spermatozoa during freezing and thawing is affected by many factors, including the composition of the cryodiluent (Curry, 2000). However, frozen-thawed semen produced reactive oxygen species (Alvarez & Storey, 1992) and excessive formation of reactive oxygen species during cryopreservation processes and have been associated with a decrease in the quality of thawed spermatozoa (Chung *et al*., 1998). Due to the availability of the some components found in *Saccoglotis. gabonensis* which are also present in *S. bicolour* there are the possibilities that "Umqombothi could be used successfully as semen extender.

According to Taylor (1998), "Uqombothi", or South African homebrew beer, is made from malted sorghum, which is a good source of lactobacilli (lactic acid fermentation caused by microorganisim of genus microbacillus under anaerobic conditions), which enhance souring from the lactic acid produced which is usually highly acidic (with low pH) and sour. Uqombothi is an unstrained fermented thin porridge that has been doubly fermented through lactic acid fermentation (*Lactobacillus leichmannii* inoculated) followed by yeast (*Saccharomyces cerevisiae*) fermentation. According to Awika and Rooney (2004), phenolic compounds affect the rate of fermentation, quality and stability of opaque beer produced from *S. bicolour* (Bvochora *et al*., 2004). The stages of traditional "Umqombothi" preparation involve the cooking of cereal meal; lactic acid fermentation; boiling of the lactic acid fermented mixture; first alcoholic fermentation; addition of the sweet, non-alcoholic beverage, straining; and the second alcoholic fermentation stages (Gadaga *et al*., 1999; Bvochora & Zvauya 2001). Studies carried out to determine the effects of fermentation on the phenolic compounds of sorghum have mainly concentrated on the high molecular weight phenolic compounds (Obizoba & Atii, 1991; Nwanguma & Eze, 1996)

although the low molecular weight phenolic compounds may have a role in the overall quality of the beer.

According to Rogel-Gaillard *et al*. (2001), several scientific advances in gamete physiology and/or manipulation have been successfully utilised while others are just beginning to be applied at the production level. Semen extenders that permit the use of fresh semen for more than 5 days post-collection are largely responsible for the success of AI in pigs worldwide. Transfer of the best genetics has been enabled by use of AI with fresh semen, and to some extent, by use of AI with frozen semen over the past 25 years. Sexed semen, now a reality, has the potential for increasing the rate of genetic progress in AI programmes when used in conjunction with newly developed low sperm number insemination technology.

The ability to cryopreserve spermatozoa from all of the domestic species is challenging. Even though all of the cells must endure similar physical stresses associated with the cryopreservation processes, sperm from the different species are very different in size, shape and lipid composition, all of which affect cryosurvival of spermatozoa. Thus, when a cryopreservation protocol is optimised for sperm of one species, it may not be ideal for sperm of other species.

Semen preservation and artificial insemination could become powerful tools for the genetic management of pig husbandry breeding programmes since these assisted reproductive techniques would allow the storage of semen from genetically valuable animals, extend generation times, circumvent husbandry or health problems that may prevent certain animals from breeding, and may facilitate transfer of semen between subpopulations that are geographically or biologically isolated (Wildt, 1992).

The success rates of AI are higher for chilled semen than for frozen semen when equally good methods for timing of the estrous cycle, and for AI, are used (Wildt, 1992). A limitation for the use of chilled semen is the survival time of the preserved spermatozoa. Means of prolonging their survival are the addition of suitable extenders, providing energy, maintaining pH and osmolarity, and protecting the acrosome and plasma membrane integrity against damage, while chilling lowers sperm metabolism. The sperm membrane contains high levels of polyunsaturated fatty acids that are highly susceptible to oxidative damage. Oxidative stress during cold shock and freezing damages the sperm membrane and reduces sperm viability (Alvarez & Storey, 1992).

1.2 MOTIVATION FOR THE STUDY

Artificial insemination (AI) has been one of the techniques used in order to improve reproductive efficiency of male animals. Nevertheless, state-of-the-art in AI techniques has not been well practised in the South African pig industry. The reason was that conventional semen extenders are very expensive and beyond the reach of small-scale farmers. Moreover, a number of emerging farmers still have insufficient knowledge about the use of AI in pigs. These limitations invariably diminish the opportunity of use of large number of spermatozoa for successful insemination and subsequent fertility of the inseminated females. This study was therefore designed to evaluate the usability of “Umqombothi” *S. bicolour* as semen extender for the improvement of the viability and fertilising capacity of boar spermatozoa.

1.3 PROBLEM STATEMENT

No confirmatory record is available on the use of “Umqombothi” (*Sorghum bicolour)* beer as boar semen extender. More importantly, the importation duties on the conventional semen extenders are very high and beyond the affordability of the local pig farmers. The problem with most of the conventional semen

extenders is that they are mostly developed for bovines rather than for pigs and they are also designed to accommodate semen life-span of more than 16 hours, which appears to be disadvantageous to boar semen storage (Chung *et al*., 1998). Conventional boar semen extenders give low artificial insemination doses per ejaculate due to low cell survival, resulting in both low fecundity rates and low survival rates (Gillmore *et al*., 1998).

Boar spermatozoa preservation has not been as extensively developed as that for the bovine. Perhaps the sensitivity of boar semen to low temperature, stressor and extenders remain a challenge. It is in the quest of finding cheaper and affordable as well as the most appropriate media for extending the storability of boar semen that this study is being proposed.

1.4 PROJECT RATIONALE

The inability to store boar semen extended in a liquid form for several days without a significant reduction in fertility (Umesiobi; 2000a; Umesiobi *et al*., 2000) severely limits the utilisation of artificial insemination in pigs. It is important to utilise the available boar spermatozoa as effectively as possible in AI in order to achieve high fertilization rates. The study is intended to use “Umqombothi” *Sorghum bicolour* to improve the viability and fertilizing integrity of boar spermatozoa as a semen extender. It is well known that sorghum is a good source of energy, and the idea is to preserve the spermatozoa in a medium in which they can survive by the inclusion of energy. The principal advantage of using unfrozen, liquid semen is that fertility is maintained even with low numbers of spermatozoa in the inseminate (Chung *et al*., 1998) but fertility of liquid semen is lost during extended periods at ambient temperatures. The main reason why the use of “Umqombothi” as a semen extender should be encouraged is that it can be relatively affordable and easy to prepare (Crabo, 1991). Another advantage of using “Umqombothi” as semen extender in this case is that it can be readily available and this can compensate for the unavailability of

conventional semen extenders because it can be easily prepared locally. The use of “Umqombothi” as a semen extender should be encouraged as it contains some similar chemicals which are contained in *Saccoglotis gabonensis*.

1.5 AIMS AND OBJECTIVES

The primary objective of the study is to:

Evaluate the suitability of “Umqombothi” *Sorghum bicolour* extender as a means of improving the viability and fertilizing integrity of boar spermatozoa.

1.6 SPECIFIC OBJECTIVES

1.6.1 To determine the effects of “Umqombothi” *Sorghum bicolour* extender on the viability of spermatozoa.

1.6.2 To determine the effects of “Umqombothi” *Sorghum bicolour* on semen and subsequent fertility of artificially inseminated sows.

1.7 HYPOTHESIS

1.7.1 Extending boar semen using “Umqombothi” (*Sorghum bicolour)* extender will improve the viability and fertilizing integrity of boar spermatozoa.

1.7.2 Time of semen collection will affect the viability of boar semen.

Chapter 2

Literature review

2.1 INTRODUCTION

The preservation of boar semen is different from the preservation of that of other domesticated mammals, primarily due to the higher sensitivity of boar sperm to chilling, freezing and thawing. Therefore, the majority of extended semen doses are used for AI on the day of collection, although some are used up to 3–5 days post-collection (Johnson *et al*., 2000), although Gry *et al.* (2004) concluded that extended liquid boar semen may be used for insemination for up to 5 days after collection. One of the main deterrents to the widespread use of artificial insemination in pigs is the short storage life of boar spermatozoa. Storage of semen at 5 to 8°C for more than 24 h causes lowered conception and embryo survival rates (Barth, 1992).

Farrowing rates are expected to be 80–85% when extended boar semen is used 48:00 post-collection and a further reduction in the farrowing rate can be expected when using 5-day-old semen (Johnson *et al*., 2000). Litter size is also reduced when using extended boar semen stored for about 3 days (Waberski *et al*., 1994c; Christensen *et al*., 2004). Dilution of boar semen presumably reduces proteins and natural antioxidants along with other components in the seminal plasma, which are required for the normal function and membrane integrity of the sperm (Johnson *et al*., 2000). During storage of extended boar semen, a reduction in the fertility potential due to sperm ageing cannot be prevented. However, handling, dilution and storage procedures may be improved in order to limit a further decrease in the fertility potential. Some studies have compared different extenders and the changes in semen quality were measured as changes in motility, pH, viability, and bacterial growth, during storage (Waberski *et al*., 1994b). These functional and structural changes are regarded as part of

the natural ageing process of the sperm, which may be affected by the dilution conditions and the time of storage (Johnson *et al*., 2000). The use of semen extenders in the pig industry has been in existence for quite a number of years. However, researchers and farmers are still striving for the most appropriate measures of preserving the quality of stored semen. Improving the viability and fertilizing integrity of boar spermatozoa is still a problem in the pig industry because of the limited lifespan of the boar spermatozoa (Umesiobi & Iloeje, 1999; Umesiobi, 2000b; Umesiobi *et al*., 2002; Umesiobi, 2006b). The storage tolerance of the spermatozoa without any noticeable decrease in quality depends, among other factors, on the choice of the extender (Levis, 2000). The improvements of boar semen preservation techniques require reliable methods for the study of the differences in the semen quality following different treatments, such as the use of different semen extenders (Waberski *et al.,* 1994b). One such semen extender is “Umqombothi” which is home-brewed sorghum beer in South Africa. Until recently, the effects of storage on the DNA integrity in extended liquid boar semen had not been studied. Mature sperm cells apparently have a lack of DNA repair mechanisms. Therefore, a sperm cell’s defence against damage to the DNA is dependent on two factors. Firstly, the tight structure of the sperm chromatin, resulting in DNA in sperm being six times as condensed compared to somatic cells. Secondly, antioxidants in the seminal plasma protect the sperm against oxidative damage (to sperm membranes and DNA) by reactive oxygen species (ROS). The damage that ultimately occurs to the DNA of the sperm, despite the defence mechanisms can only partially be repaired by the zygote after successful fertilization (Ahmadi & Ng, 1999). The sperm chromatin structure assay (SCSA) is a flow cytometric method that utilises the metachromatic properties of the dye acridine orange (AO), staining single-stranded DNA red, and double-stranded DNA green (Evenson *et al*., 2002). The assay detects susceptibility to sperm DNA denaturation *in situ* and is objective, precise, fast, and can be performed on frozen-thawed samples (Evenson *et al*., 1994). Furthermore, the SCSA has been used on a number of different species

(Evenson *et al*., 2002) and fertility data have been shown to correlate with the results obtained from the SCSA of human (Evenson *et al*., 1994; 2002).

2.1.1 SEMEN CHARACTERISTICS

Semen is a white or grey liquid, emitted from the urethra (tube in the penis) on ejaculation. Examination of semen characteristics, such as sperm morphology, concentration and progressive motility, are routine procedures for obtaining information about potential male fertility in AI centres. However, these semen traits were often not significantly correlated to fertility (Barth, 1992). In commercial centres, semen assessment generally includes the evaluation of ejaculates characteristics such as sperm concentration, morphology, viability and motility, although some of these characteristics can be used to detect male reproductive disorders that result in low fertility, they are not useful in predicting relative fertility in healthy boars with ejaculate quality that meets normal industry standards (>70% motility and <30% abnormal sperm), even though the productivity of the boars substantially differs (Ruiz-Sànches *et al*., 2005).

Mixing semen from several boars (i.e. heterospermic semen) in the same AI dose may increase the reproductive performance compared to the use of semen from only one boar i.e. homospermic fertilization (Cevorsky *et al*., 1999).

Semen characteristics are exemplified by semen volume, semen colour, sperm motility, sperm concentration per ml, sperm concentration per ejaculate, live sperm, normal sperm, sperm morphology, and semen pH.

2.1.1.1 Semen volume

Semen volume is the quantity of the seminal fluid which contains sperm cells and gelatinous, and is produced per ejaculate by a male following a successful ejaculation. Usually each millilitre of semen contains millions of spermatozoa, but

the majority of the volume consists of secretions of the glands in the male reproductive organs. The normal whole boar ejaculate must be of considerable volume (>150 ml) to ensure fertilization. Quantities below this range can have a negative impact on fertilization rates. The purpose of semen is purely for reproduction, as a vehicle to carry spermatozoa to the female reproductive tract. It is essential to determine sperm concentrations in order to rank individual males and or to calculate appropriate levels of dilution (Rigau *et al*., 1996).

Several researchers have studied the effect of semen volume and concentration on fertility. Zahraddeen *et al*. (2005) found no relationship between sperm concentration and fertility. However, Dumpala *et al*. (2006) found a significant relationship between sperm concentration and fertility. Nevertheless none of these reports found a significant positive correlation between fertility and semen volume. During a fertility study where hens where artificially inseminated with 0.025 ml cock semen bi-weekly, Zahraddeen *et al*. (2005) reported a correlation between fertility and semen volume. In addition, a significant ($P \leq 0.05$) relationship between fertility and sperm concentration was found to exist. The significant correlation between fertility and semen volume found by Hazary *et al*. (2000) suggested that males could be selected for fertility midway through the breeding season based on semen volume.

2.1.1.2 Semen colour

Even though microscopic evaluations are the standard for accepting or rejecting ejaculates, it is important not to forget obvious visual and olfactory characteristics of semen (Rozeboom, 2001). The normal boar ejaculate should be a milky white colour. Nevertheless, in some instances, the normal boar semen colour can be somewhat yellowish, but normally it has a similar appearance to that of skim milk, and occasionally with small amounts of blood, usually originating from the urethra, may be present in the ejaculate, which gives the semen a pinkish hue colour (Rozeboom, 2001). This abnormality does not reduce the fertility or the

viability of the ejaculate (Roldàn, 1998), but a darker red colour is usually associated with a pungent odour and thus should be cause for discarding the collection (Rozeboom, 2001). To a certain extent semen colour can be an appropriate estimate for determining sperm concentration, since light milky colour is associated with low sperm concentration and the darker skim milk colour is associated with high sperm concentration under normal circumstances (Roldàn, 1998).

2.1.1.3 Sperm motility

Sperm motility has long been considered a major criterion in the assessment of male fertility (Haugan *et al*., 2004); the objective of estimating sperm motility is to determine the motile proportion of spermatozoa and the proportion moving progressively, i.e., actively moving forward. Traditionally, the evaluation has depended on subjective estimates of sperm motility characteristics using a microscope. This method is cheap and simple to use; however, it has the disadvantage that sperm motility estimates can vary among examiners, who can be biased in a number of ways (Malmgren, 1997). For example, the examiner may have prior knowledge of the boar's fertility, or may have viewed its previous ejaculates. Ejaculated boar spermatozoa are vulnerable to cold shock and prolonged storage of boar spermatozoa at low temperatures reduces survival rate resulting in bottleneck for the extension of artificial insemination in pig husbandry (Haugan *et al*., 2004). Interestingly, sperm motility could be considered as a functional marker in boar sperm analysis, since motility is directly related to the sperm's ability to obtain and process energy (Roldàn, 1998). Sperm motility is an important trait as it is a factor which allows the sperm cell to travel to the zona pellucida.

Sperm motility is an important parameter for fertility and the molecular mechanisms of mammalian sperm motility are still largely undefined (Huang *et al*., 1996). However, an accurate determination of boar sperm motility is

troublesome (Gadea *et al*., 1998; Sànches, 1991), since the motion characteristics of these cells make an accurate subjective estimation of the samples difficult. Thus, subjective determination of boar sperm motility seems not to be a very useful tool for semen quality analysis (Rigau *et al*., 1996).

The physiological factors regulating sperm motility include protein kinases, phosphatases, calcium ion intracellular pH (Gagnon, 1995; Lanzafame *et al*., 1994). The molecular mechanisms and the signal transduction pathways mediatates the processes of capacitation and acrosome reaction are only partially defined, and appear to involve modifications of intracellular calcium and other ions, lipid transfer and phospholipid remodelling in sperm plasma membrane as well as changes in protein phosphorylation (Rigau *et al*., 1996).

2.1.1.4 Sperm concentration per ml

Sperm concentration per ml refers to the number of sperm cells and seminal fluids present in an ejaculate per ml. A greater number of sperm cells increase the chances of fertilization (Rigau *et al*., 1996). Either raw or extended semen can be used, but unextended semen has disadvantages, such as a tendency to agglutinate, in which case if the sperm concentration is high the estimated percentages of motile spermatozoa may become higher (Willenburg *et al*., 2003). *In vitro* storage of semen leads to low survival of sperm cells. Extending semen prevents the agglutination of spermatozoa and reduces the influences of sperm concentration and seminal pH (Malmgren, 1997). To obtain an optimal subjective estimate of sperm motility, it is recommended that the semen be diluted in an appropriate extender to a constant sperm concentration (25 to 50 x 10^6/ml) (Umesiobi, 2007). The use of a phase-contrast microscope (magnification x 200 to 400) facilitates viewing of the spermatozoa. Furthermore, it is important to carefully control the temperature of the equipment used to prepare the slide as well as the temperature of the slide during viewing (Malmgren.1997). Before AI is executed it is imperative to note that semen of good quality and quantities should

be available in order to facilitate the AI process effectively, but not only the quality should be taken into account in this manner. Sperm concentrations are important as they are also required for successful inseminations. According to (Willenburg *et al*. 2003), the minimum number of spermatozoa required for successful insemination should be (2 x 10^9 spermatozoa/ml), however Huang *et al*. (2004) found that increasing the number of spermatozoa can reduce the loss of fertilizing capacity associated with storage. Nevertheless, in many of the field trials recently conducted by Sancho *et al*. (2004), ejaculates are evaluated for motility, morphology and concentration diluted to 1.5 X 10^6 sperm per ml extender.

Willenburg *et al*. (2003) reported that optimal conditions for fertility may include sperm concentration of 3 X 10^9 fertile sperm. Whatever the influence of individual features and breed, a positive correlation exists between sperm concentration and testicular activity (Borg *et al*., 1993; Ciereszko *et al*., 2000).

2.1.1.5 Sperm concentration per ejaculate

Sperm concentration per ejaculate which gives an indication of the total number of spermatozoa present per ejaculate is important as it is also a parameter which plays a greater role in the accomplishement of successful inseminations in pigs. Sperm concentration is also reported to be positively correlated with conception rates and litter size (Umesiobi, 2009). According to Umesiobi & Iloeje (1999) and Umesiobi (2006a), the minimum number of spermatozoa required for successful insemination should be 2.5 x 10^9 spermatozoa per ejaculate.

Generally, there are four basic parameters that are measured to evaluate boar semen quality. These include sperm concentration, sperm motility, sperm morphology and sperm acrosome integrity. Of these, sperm concentration and motility are perhaps most routinely used for sorting ejaculates prior to processing, since they require the least amount of time and are required to calculate semen

doses per ejaculate (Rozeboom, 2001). However, in the study conducted by Willenburg *et al*. (2003), the optimal conditions for fertility may include sperm concentration of 3 X 10^9 fertile spermatozoa per insermination. It is however, very important to note that measuring sperm concentration or total numbers of spermatozoa is not a component of semen quality evaluation, but more so, as a tool to monitor the health and productive output of the boar and as the primary feature in processing boar ejaculates. For optimising the genetic potential of a single individual, accurate assessment of sperm numbers is not the only factor for increasing semen doses per ejaculate and boar stud efficiency in terms of semen output (Rozeboom, 2001).

Sperm concentration affects the amount of seminal plasma surrounding each spermatozoon, both in raw and extended semen. As sperm concentration increases, the amount of seminal plasma per sperm cell decreases (Kommisrud *et al*., 2002).

Motility is important for semen quality; however motility alone does not secure fertilizing capacity (Kommisrud *et al*., 2002). Spermatozoa also need intact acrosomes to penetrate the barriers around the ovum. The results from the trial indicate that the acrosome is more susceptible to damage during storage than the organelles being the structural basis of motility (Kommisrud *et al*., 2002). This presumption is in accordance with the report of Buhr (1990) which indicated that the decrease of membrane fluidity during storage is greater for head plasma membranes than for sperm body membranes. This is not surprising as storage of diluted semen to some extent may cause sperm capacitation possibly followed by acrosome-reaction (Vishwanath & Shannon, 1999). The decrease in acrosome integrity might thus be due to acrosome reaction in addition to membrane damage (Kommisrud *et al*., 2002). When looking at semen quality during storage one should not put too much emphasis on motility estimates alone, but also give attention to other quality parameters to get as close to fertilizing capacity as possible (Kuster & Althouse, 1999). Looking at the different

factors that might have an influence on motility and acrosomal integrity during storage, the particular study reveals a significant influence of boar on semen viability. The influence was evident for both motility and acrosome integrity (Kommisrud *et al*., 2002). On the other hand, the dependent variables were not affected by breed of the boars.

The sperm concentration per ejaculate seems, however, to play an important role for motility but not for acrosome integrity during storage (Kommisrud *et al*., 2002). The fact that the regression coefficient for sperm concentration in the statistical analysis is negative, demonstrates that the motility is maintained at a higher level during storage when sperm concentration in undiluted semen is low compared to higher sperm concentration. This suggests that there is a positive effect of increasing the amount of seminal plasma and furthermore that there might be components in seminal plasma which are beneficial for maintenance of motility, and that the concentration of these components after extension might be important (Kommisrud *et al*., 2002). This presumption is in accordance with results from a study comparing 2 extenders for long-term storage of boar semen, showing one extender to give fecundity of sperm cells superior to the other (Kuster & Althouse, 1999). The positive effect of additional seminal plasma on viability of bull spermatozoa during extreme extension has been demonstrated by Garner *et al*. (2001). Results of the investigation reveal that the effect of boar semen is of great importance concerning semen quality during longtime storage. Further, there seems to be a beneficial effect in increasing the amount of seminal plasma on motility.

2.1.1.6 Normal sperm

The male gamete produced in the testis is a highly polarised but functionally immature spermatozoon that requires further differentiation in the epididymis to become progressively motile and to acquire fertilizing capacity (Olson *et al*., 2002). This crucial developmental process requires sperm interaction with a

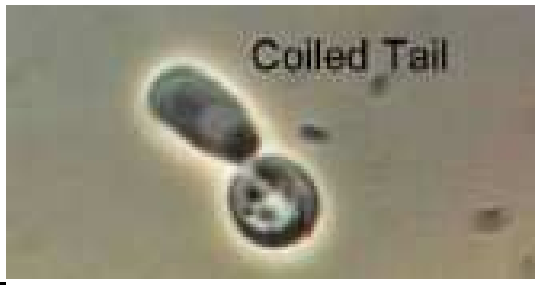
progressively changing luminal environment regulated by region-specific secretory and absorptive activities of the epididymal epithelium (Hinton & Palladino, 1995). The environment of the spermatozoa in the epididymis is definitely the most complex fluid found in any exocrine gland. This complexity is the result of two particularities: (1) continuous and progressive changes in its composition along the duct and (2) the presence of unusual concentrations of several components (some of which are not found in any other body fluids) (Olson *et al*., 2002). This specificity is maintained not only by active secretion and re-absorption along the tract, but also by the presence of significant restrictions in the exchanges between the luminal compartment and blood plasma (Cyr *et al*., 2002).

2.1.1.7 Sperm morphology

Sperm morphology (Haugan *et al*., 2004), and particularly the acrosome status (Yanangimachi, 1994) is an important indicator of fertility. Few large quantitative studies on acrosomal morphology have been conducted in conjunction with breeding trials because assays of morphology of a hundred sperm cells are labour intensive, but new emerging technology to assess sperm motility rapidly and objectively can overcome the labour problem (Gravence *et al*., 1999).

In the study conducted by Kuster and Althouse (1999), the authors recommended that storage of boar semen be no longer than three days. Sperm morphology and acrosome integrity are also effective tools to estimate semen viability and can also provide more information about the ejaculate in terms of its quality than is possible with just a motility evaluation. Still both of these criteria are important to use along with motility, as a determinant for keeping or discarding ejaculates (Kuster & Althouse. 1999). Morphologically abnormal and poorly motile sperm can fertilize eggs and sperm without intact acrosomes cannot fertilize eggs because acrosome integrity plays a privital role in the fertilisation of the egg. Boar stud farmers who do not evaluate all three of these

semen quality components possibly underestimate the true fertility potential and quality of an ejaculate (Kuster & Althouse. 1999). The initial processing level for normal spermatozoa was higher than 70% normal morphology when semen is used after extended storage lengths (>24 hours) (Kuster & Althouse. 1999).



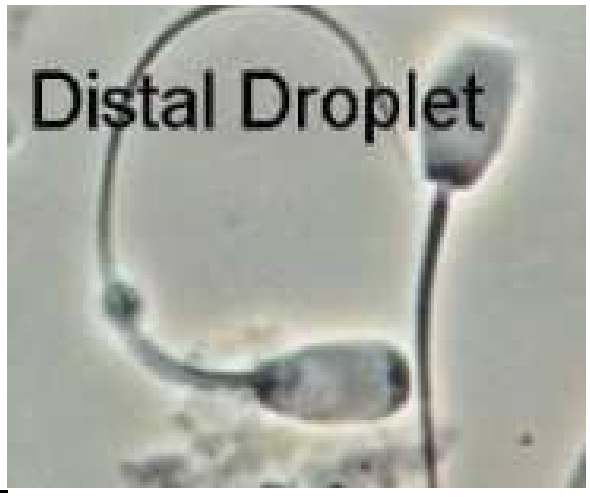


Figure 2.1 Common sperm head, mid piece, tail and acrosome abnormalities (Kuster & Althouse. 1999).

The figure above shows some common sperm acrosome abnormalities observed in the study conducted by Kuster and Althouse (1999) where the authors extended boar semen in Beltsville Thawing Solution and Cornell University Extender to determine the course of acrosome abnormalities in boar semen. Examination of semen involved thin smearing of semen stained with an eosin/nigrosin stain, which can also be used to determine the percentage of live/dead sperm. This determined the percentage of abnormal sperm (head, body and tail defects) which were determined under the phase contrast microscope. These may account for 0 – 20% of total count in a normal semen sample.

In the study conducted by Sancho *et al*. (2004) on post-pubertal Landrace boars, significantly lower frequencies of mature and immature spermatozoa with distal cytoplasmic droplets and significantly higher frequencies of immature spermatozoa with proximal droplets were observed in boars exposed to a decreasing photoperiod (Sancho *et al*., 2004). This result indicates that sperm fertility of boars decreases during decreasing photoperiod, in comparison with increasing photoperiod, mainly due to impaired testicular function (Sancho *et al*., 2004).

In humans, cholesterol in seminal plasma is claimed to inhibit spermatozoa from undergoing acrosome reaction and to improve survival (Cross, 1996). The membranes of boar spermatozoa consist of relatively low amounts of cholesterol, particularly in comparison with those of humans (De Leeuw *et al*., 1990), and one might expect corresponding conditions in seminal plasma. The possible low cholesterol content of seminal plasma, which is even reduced during semen extension, could explain why there seems to be no influence of sperm concentration on preservation of acrosome integrity during storage (Kommisrud *et al*., 2002). There might be variation in intrinsic properties of the membranes, possibly being of significance to sperm membrane functionality (Gadella *et al*., 1999) which might explain the differences between individuals. There is no

influence of weight of the ejaculate on the semen quality parameters investigated during storage in this trial (Kommisrud *et al*., 2002).

2.1.1.8 Semen pH

Regulation of the function of spermatozoa, such as initiation of the acrosome reaction or motility is associated with changes in the internal pH of the spermatozoa (Sase *et al*., 1995; Umesiobi, 2007). A rise in external pH followed by an increase in internal pH initiates motility of boar spermatozoa (Gatti *et al*., 1993) and alkalinisation of the extracellular fluid alone can induce the re-initiation of spermatozoa (Saito *et al*., 1996). Extended day lengths of spring and reduced day lenghts of autumn do not affect semen volume, but result in an altered semen pH in selected boars (Sancho *et al*., 2004). A pH meter is normally used for the evaluation of semen pH.

2.2 FACTORS THAT AFFECT BOAR SEMEN VIABILITY

2.2.1 Boar effect

According to Foxcroft *et al*. (2008) the reproductive efficiency of the pig herd is highly correlated with the reproductive capacity (fertility) of the males. Poor quality boars, because of the polygamous structure of pig production, will affect the reproductive outcome of numerous females; from this it is clear that the reproductive efficiency of the boar plays a vital role in the genetic make up of the the herd. Using sub-fertile boars and low quality ejaculates for AI reduces production efficiency and lowers profit margins for the producers.

Boars are recognised as a significant source of variation with regard to the success of both *in vivo* (Flowers, 1997; Xu *et al*., 1998) and *in vitro* (Xu *et al*., 1996) fertilization in pig. This can be attributed to many factors such as breed, age, and environment. Numerous studies have shown relationships between

semen quality estimates such as motility, morphology, and viability, and fertility estimates such as farrowing rates, numbers of pigs born alive, and *in vitro* fertilization rates in order to develop procedures for selecting boars prospectively for use in both systems. Notwithstanding that reports drawn from other studies are equivocal, in some cases, characteristics such as normal acrosomes (Xu *et al*., 1998), normal head and tail morphology (Gadea & Matas, 2000), and progressive forward motility (Ivanova & Mollova, 1993; Flowers, 1997) had a positive relationship with boar fertility, while in others they do not (Xu *et al*., 1996; Flowers, 1997). Observations from field studies demonstrate clearly that most estimates of semen quality and fertility vary significantly for boars over time (Flowers, 1997, 1998). Consequently, it is also possible that relationships between these two groups of measurements are not the same for all boars. In other words, a given semen quality estimate may be a good predictor of *in vivo* or *in vitro* fertilization for one boar, but not for another (Popwell & Flowers, 2004). However, it is important to note that no breed excels in all basic semen characteristics (i.e. volume, concentration, motility and proportion of abnormal spermatozoa). For example, in a Canadian study comparing semen characteristics of five breeds, Hampshire boars showed the largest semen volume, Duroc boars were best in sperm concentration and Yorkshire boars had the highest motility score (Gadea & Matas, 2000).

Monitoring of semen quality is the first step towards the improvement of pig fertility (Tardif *et al*., 1999). According to Rothschild (1996), differences in boar fertility are mainly due to genetics and not only due to environmental effects. The detection of these differences in boar fertility is very important for pig producers because the impact of males on herd reproduction performance is high, particularly when artificial insemination is used (Juonala *et al*., 1998). There is a diversity of opinions on the optimal ambient temperature for boars. Some authors recommend 16–18°C (Baltàì Moner, 1997), and others 16–28°C (Van Groenland, 1993). It has been established that the Large White breed can produce normal spermatozoa at an ambient temperature of 16–29°C (Gadea & Matas, 2000).

In a field study conducted by Corcuera *et al*., (2002) it was observed that boars housed on concrete floors and straw bedding produced semen with high percentages of normal acrosomes during summer and optimum motility during warmer seasons of the year. On the other hand, Popwell and Flowers (2004) reported that the suitability of this strategy relied on the variability in semen quality parameters that normally occurs in an individual boar over time. When comparisons were made among boars, farrowing rates, numbers of pigs born alive, and monospermic penetration rates were significantly different, but progressive motility, normal head and tail morphology, and acrosome morphology were not. However, when comparisons were made among ejaculates within individual boars, there were significant effects of semen quality on both *in vivo* and *in vitro* fertility, in the same study.

These results demonstrate that simply relying on the means of common semen quality estimates from some boars has limited value in terms of being used as a prospective indicator of their *in vivo* or *in vitro* fertility. In contrast, characterisation of relationships between semen quality and fertility estimates is useful for estimating differences in the fertility of ejaculates from individual boars. However, both quantitative and qualitative differences in these relationships among boars are present and a given semen quality estimate that is a good predictor of *in vivo* or *in vitro* fertilization for one boar, may not be applicable for others (Popwell & Flowers, 2004).

Previous studies by Flowers (1997) highlighted those improvements in farrowing rate as the proportion of spermatozoa exhibiting progressive motility or having normal acrosomes in an ejaculate increases. However, Braundmeier *et al*. (2002) reported that detecting differences in farrowing rate may be more difficult than finding those associated with litter size in boars. Alternatively, Xu *et al.* (1998) were not able to detect differences in numbers of pigs born alive among boars when of 3 X 10^9 fertile spermatozoa were inseminated. However, reducing the

number of sperm cells to 2 X 10^9 produced sufficient results to rank boar fertility based on litter size (Popwell & Flowers, 2004).

Fertility is one of the most important economic traits in pig production and reproductive performance is controlled by the genetic make-up of the dam, boar and offspring: overall, it is largely affected by the environment (Lin *et al*., 2006). Implementation of AI in pig production allowed improvement in selection of the boars for production traits. There is evidence to support the concept that boars exhibit fertility patterns based on the number of spermatozoa inseminated and that these differ among individuals (Flowers, 2002). Two assumptions central to the existence of fertility patterns are that males differ in their fertility when the same number of sperm are inseminated and that increasing the number of spermatozoa inseminated increases fertility within some portion of the fertility curve (Johnson *et al*., 2000). Accordingly, Flowers (2002) observed that there are two basic characteristics that are directly responsible for a boar's influence on litter size: the number of spermatozoa inseminated and the proportion of these that can successfully engage ova. Consequently, it is unlikely that the observed differences in litter size were due to individual variations in these characteristics (Flowers, 2002). It therefore appears obvious that the male is responsible for multiple pregnancies per year in natural services and hundreds or even thousands of pregnancies as a result of AI, and because of the major impact of individual male on multiple pregnancies and ability to estimate fertility of male more accurately than of females (Umesiobi & Iloeje, 1999; Umesiobi, 2006a, b), more emphasis is placed here on evaluating boar semen, despite the fact that the sows are the major contributors in each reproductive cycle (Foote, 2003; Umesiobi, 2008,c). Therefore to fully utilise semen from proven boars, adequate breeding strategies need to be established in order to prolong boar semen durations without negatively affecting its viability.

Considerable variations have also been observed among boars concerning the fertilizing capacity of semen during storage (Waberski *et al.,* 1994c). Several

other factors have also been implicated to influence fertility of stored semen (Kommisrud *et al*., 2002). Individual variation concerning the chemical composition of the ejaculate as well as the amount of seminal plasma might be of importance (Kommisrud *et al*., 2002). Seminal plasma is important for progressive motility of sperm cells. Spermatozoa gain motility during ejaculation as pH and bicarbonate concentration increase during mixing of sperm and seminal plasma (Rodriguez-Martinez *et al.,* 2001).

2.2.2 Semen collection and management

Semen is normally collected by an experienced operator using the gloved-hand technique, preferably by the same operator in order to reduce variations (Huang *et al*., 2004), then filtered through a double layer of cheese cloth to remove gel (Kozink *et al*., 2002), or it could be collected in a room equipped with an artificial sow. Thereafter it should be evaluated for motility and for morphology by a suitable method. Inexperienced boars should be trained to mount the sows for successful semen collection, and they should again be moved into the room equipped with artificial sow to ensure that they are actually trained (Kozink *et al*., 2002; Umesiobi *et al*., 2002, 2004). However if the boar fails to mount the artificial sow and ejaculate within 10 min, the animal should receive an appropriate treatment and additional 10 min of exposure to the artificial sow should be allowed (Kozink *et al*., 2002). The reason for this could be that it would have to get used to the presence of the sow or to the stimulation of testosterone. According to Huang *et al*. (2004), treating young and sexually inexperienced boars with $PGF_{2\alpha}$ improves libido and increases the number of boars mounting an artificial sow during the first or the second exposure (Kozink *et al*., 2002).

According to Kommisrud *et al*. (2002) boars do not exhibit sexual motivation combined with ejaculation until between the age of 150 and 270 days. This implies that the age for semen collection should also be borne in mind where AI is practised. In the study conducted by Willenburg *et al*. (2003), semen ejaculates

are immediately collected into a cup and evaluated and extended to a proper concentration based on the concentration, motility and abnormalities. Normally after semen collection semen stored in an appropriate container is held in the water bath to maintain 37°C taken to the laboratory and then evaluated for all the parameters namely: semen pH, sperm motility, sperm concentration and the morphology. After the ejaculation is extended to appropriate concentration, it is divided into aliquots, sealed and allowed to cool to room temperature. The percentage values of abnormal sperm, acrosome morphology and the pH levels are highly influenced by the rate of fructolysis, which involves the breakdown of seminal fructose by sperm to generate energy for motility (Umesiobi, 2004; Umesiobi *et al*., 2004).

Table 2.1 Minimum procedures and equipment for semen quality evaluation of boar ejaculates following collection and prior to processing (Rozeboom, 2001).

Evaluation Procedures	**Equipment Needed**[a]
1a. Visual and olfactory assessment of ejaculate	None
1b. Determine semen volume and sperm concentration	Balance and a haemacytometer or photospectrometer
2. Motility	
a. Prepare a 1:10 dilution of semen with semen extender.	
b. Gently rotate the semen.	
c. Remove a small sample (5 to 10 ml) and place in a clean glass test tube.	
d. If necessary, warm it to 36 to 37 degrees centigrade (body temperature).	Small Water Bath
e. Place a small drop on a pre-warmed slide and gently place a cover slip over the drop.	Slide Warmer
f. Immediately examine the sample at 100x and then at 400x.	Self illuminating microscope capable of 100x, 400x, magnification and glass slides with coverslip
g. Estimate the percentage of sperm in fields that are progressively motile	

h. Examine several fields and establish an average.	
i. Record your estimate to the nearest 5 or 10 percentage units.	Small, disposable plastic pipette
3. **Morphology**	
a. After the motility estimate is complete, allow the slide to cool. Motility will slow or stop and individual sperm cells can be observed. or Prepare a stained semen sample-using step 4a, with a mixture (1:1) of morphology stain and formal saline.	Self-illuminating microscope capable of 100x and 400x and 1000x (oil) magnification; glass slides and immersion oil Eosin-nigrosin stain
b. Switch to the 400x objective and observe individual cells in several fields.	
c. Estimate, in several fields, the percentage of cells that are "normal" (see example pictures).	
4. **Acrosome integrity**	Self illuminating phase contrast microscope capable of 100x, 400x, 1000x (oil) magnification
a. From the same semen sample in step 1a, prepare a 1:1 dilution of semen and a mixture (1:1) of formal saline and Acrosome stain on a glass slide.	Formal saline: 6.19g $Na_2HPO_32H_2O$: 2.54g KH_2PO_4: 4.41g NaCL: 125 ml 38% formaldehyde: 1000 ml distilled water. naphthol yellow or erythrocin stain
b. Place one or two drops of semen and 1-2 drops of the stain mixture on a glass slide and mix gently with the tip of the pipette. Use the edge of a second slide to draw the mixture across the flat slide to produce a thin layer. Allow the slide to air dry.	
Place a drop of microscope immersion oil under the slide and view first at 10x to focus, and then switch to either 40x or 100x and view individual cells. (Be sure that you don't get oil on non-oil lens.)	
d. Estimate, in several fields, the percentage of cells that are "normal".	

2.2.3 Duration of semen storage

The duration for the storage of boar semen has remained a question for many years, and the available semen extenders have differing storage capacities. Waberski *et al*. (1990) reported that after storage of semen for 48 to 87 hours for subsequent AI services, the fertilization rate decreased even when ovulation occurred between 12 and 24 hours after insemination. Farrowing rates were 80–85% when extended boar semen was used up to 48 hours post-collection and a further reduction in the farrowing rate was recorded when using 5-day-old semen (Johnson *et al*., 2000). Litter size was also reduced when using extended boar semen stored for 3 days (Wabarski *et al*., 1994b; Christensen *et al*., 2004).

Dilution of boar semen presumably reduces proteins and natural antioxidants along with other components in the seminal plasma, which are required for normal function and membrane integrity of the sperm (Maxwell & Johnson 1999). During storage of extended boar semen, a reduction in the fertility potential due to sperm ageing cannot be prevented. However, handling, dilution and storage procedures may be improved in order to limit a further decrease in the fertility potential. Some studies have compared different extenders and the changes in semen quality measured as changes in motility, pH, viability, and bacterial growth during storage (Webarski *et al*., 1994a).

These functional and structural changes were regarded as part of the natural ageing process of the sperm, which might be affected by the dilution conditions and the time of semen storage at 3 days (Johnson *et al*., 2000).

Results from the experiment conducted by Waberski *et al*. (1990) revealed the negative relationship between semen age and fertilization rate and the accessory sperm number. In contrast, pregnancy rates based on the ovulation

of day 2 to day 4 embryos did not decrease significantly until day 4 of semen storage, and therefore seem to be insufficient as a parameter for assessing semen quality differences due to aging. Lambert *et al*. (1991) and Waberski *et al*. (1990) reported that fertilization rates and frequency distribution of number of accessory spermatozoa were higher in semen stored in Androhep than in Beltsville Thawing Solution medium. It seems however, that apart from slight differences between media in the preservation of boar semen, the decrease in fertilizing capacity due to *in vitro* aging of spermatozoa cannot be prevented even during the first day of storage (Waberski. 1990).

According to Waberski *et al*. (1990) the use of boars with good semen quality could prevent the decline in fertility associated with storage. However practical means for evaluating semen quality in order to predict male fertility are still required. To fully utilise semen from excellent boars the techniques for extending the storage time of left-over semen must be developed (Huang *et al*., 2004). In the pig industry a single male has a more profound impact on efficiency and reproductivity than an individual female and the impact is even higher with the use of AI (Ruiz-Sànches *et al*., 2005). This is due to the fact that the male is responsible for large numbers of pregnancies throughout its life-span.

2.2.4 Storage temperature

In artificial insemination, the dilution and storage procedures represent an additional shock for boar spermatozoa. Ejaculated boar spermatozoa are vulnerable to cold shock and prolonged storage of boar spermatozoa at low temperatures reduces survival rate resulting in bottlenecks for the extension of artificial insemination in pig husbandry (Haung *et al*., 2004). As boar sperm are extremely temperature sensitive due to the lipid composition of their plasma membrane (Robertson *et al*., 1990; Parks & Lynch. 1992), the choice of the right temperature for dilution and storage may be crucial. The storage of diluted boar

semen is commonly performed at between 15 and 17 °C. However, the delivery of diluted samples, especially in winter, is subject to temperature variation. The critical role of such variation has been evaluated in only a few studies (Green & Watson. 2002). Nevertheless, De Leeuw *et al*. (1990) and Morrow (1994) found that current commercial practice is to store extended boar semen between 15 and 20°C until use for AI. A previous study by Rozeboom (2001) showed that the fertility potential of extended porcine semen stored at 15°C yields acceptable fertilization rates, while exposure to temperatures below 15°C would result in cold-shock and cell death. The proposed critical temperature (<15 °C) therefore, appears to have originated from early research (Haung *et al*., 2004) on the optimum storage temperature of unextended, fresh boar semen.

It is important to consider the impact of temperature fluctuations on sperm cells when semen is not directed into the lab right after collection (i.e. transported between barn and laboratory or to the sow house). A rapid drop in temperature results in cold shock; this is evident when sperm are initially viewed under the microscope (Rozeboom, 2001). Sperm tails will be curled around the head or tightly spiralled beneath the sperm head. Small numbers of sperm cells exhibiting this general appearance may normally be present in an ejaculate; however, more than 10% curled tails in association with a temperature of less than 32°C is a good indication of cold shock. It may not be necessary to discard this sample, but immediate dilution with extender at the same temperature to prevent further damage should be considered.

2.2.5 Climatic factors

The reproductive ability of farm animals is affected by seasonal factors such as temperature, humidity and daylight length. It is well established that reproductive efficiency in pig production is often less during and after the hot season. One reason for reduction in reproductive performance might be elevated ambient temperature, which induces heat stress. Many factors influence sperm production

such as season (Rogel-Gaillard *et al*., 2001), breed (Borg *et al*., 1993), testis size (Rogel-Gaillard *et al*., 2001), and nutrition (Louis *et al*., 1994). Gadea *et al*. (1998) and Ruiz-Sànches (1991) found no adverse effect of season on sperm production, while others, such as Colenbrander *et al*. (1993), have reported a decrease in sperm production in the spring. The effect seems not only to be due to elevated temperature, but also to cyclic variation in photoperiod (Weberski *et al*., 1990). Pigs have a low capacity for increased sweating when temperature increases, which also contributes to the close relationship between scrotal and testicular tissue temperatures during heating. Heat may adversely affect spermatogenesis, causing a mild to moderate testicular degeneration. Louis *et al*. (1994) concluded from their study that normal sperm output of Large White boars could be maintained at air temperatures as high as 29°C. Gadea & Matas (2000) found a reduced number of spermatozoa and an increase in number of abnormal spermatozoa when placing boars at 33°C for 72 h. Van Groenland (1993) exposed boars to 35°C for 100 h. This resulted in a decreased semen quality with increased percentage of abnormal spermatozoa but ejaculate volume and total sperm count per ejaculate remained unaltered. Even local heating of the scrotum in Landrace boars has been reported to cause disturbances in spermatogenesis, due to degeneration of the seminiferous epithelium (Barth, 1992). However, the author did not find any consistent difference in ejaculate volume and total sperm count per ejaculate in the majority of the boars exposed to local heating of the scrotum.

Temperature is one of the factors with most influence on the reproductive indexes of a farm, determined by a thermal balance of the air (gas mixture), animals and housing facilities (Baltàì Moner, 1997). High temperatures affect the spermatogenesis, lowering the production and sperm quality (Popwell & Flower 2004). In pigs, housing facilities (such as type of floor and bedding) affect weight gain (Louis *et al*., 1994) and limb faults (Barth, 1992). There is a diversity of options on ambient temperatures for boars. Some authors recommend 16-18°C (Baltàì Moner, 1996), and some 16–28°C (Van Groenland, 1993). It has been

established that the Large White breed can produce normal spermatozoa at an ambient temperature of 16–18°C (Baltàì Moner, 1997).

The reproductive ability of farm animals is affected by seasonal factors such as temperature, humidity and daylight length. It is well established that reproductive efficiency in pig production is often less during and after the hot season. One reason for reduction in reproductive performance might be elevated ambient temperature which induces heat stress (Suriyasomboon *et al*., 2004). Many factors influence sperm production such as season, and breed (Borg *et al*., 1993), testis size (Huang & Johnson, 1996), and nutrition (Louis *et al*., 1994). However Baltàì Moner (1997) found no adverse effect of season on sperm production, while others like Colenbrander *et al*. (1993) have reported a decrease in sperm production in the spring. The effect seems not only to be due to elevated temperature, but also to cyclic variation in photoperiod (Chung *et al*., 1998). In the selection of the facilities for studs, one must bear in mind the climate characteristics of the location. Pig AI is increasing rapidly with the ability to retain fertility of semen stored for several days at about 18°C (Johnson *et al.,* 2000).

Mammalian seminal plasma (SP), a physiological secretion from multiple glands of the male reproductive tract, is known to both inhibit and stimulate sperm function and fertility. Certain SP proteins are able to bind to the sperm plasma membrane and play an important role in capacitation of sperm and fertilization of the egg (Yanagimachi, 1994). In previous studies, it has been reported that the ability of ram SP to repair membrane integrity of cold-shocked sperm (Chung *et al*., 1998) as well as seasonal differences in the ability of ram SP proteins to prevent cryoinjury (Peŕez *et al*., 2001) Conversely, detrimental effects of SP on sperm cell motility, viability and survival after freezing–thawing have also been reported. These contradictory results indicate that SP is a complex mixture containing a wide variety of components that affect sperm survival and motility.

Temperature has a strong effect on sperm motility (Chung *et al*., 1998). Johnson *et al*. (2000) and Chung *et al*. (1998) found that integrity and viability of spermatozoa could be preserved well during *in vitro* storage at 20°C and 15°C for 24 to 48 hours, but fertility of liquid semen is gradually lost during extended periods at ambient temperatures. Therefore the proper assessment and the viability and fertilizing ability of the spermatozoa is of the utmost importance for choosing the best way to store semen *in vitro* and for deciding how long fresh semen can be stored (Chung *et al*., 1998). In the same field trial conducted by Chung *et al*. (1998), it was found that the motility of the spermatozoa stored at 39°C was better than that of other groups in the first 8 hours, but it dropped notably thereafter and was only 34.2% by 12 h and this was due to high rate of metabolism and rapid energy consumption at 39°C (Chung *et al*., 1998). Nonetheless, in this study, temperatures of 20 and 15°C were better for maintaining sperm motility than 39 and 4°C.

Table 2.2 shows a comparision of sperm motility between semen stored at different temperatures, there was no significant difference in semen stored at 39°C, 20°C, and 15°C in the first and second hour of storage, but a significant difference was observed in semen stored at 4°C in the first and second hour of storage were the sperm motility was the lowest. During the fourth hour of semen storage no significant difference of sperm motility was observed between semen stored at 39°C and 20°C but significant difference was observed in semen stored under 15°C and 4°C, but from the eighth to forty-eighth hour no significant difference in sperm motility was observed in all the treatments.

Table 2.2 Percentage of motile spermatozoa in boar semen during *in vitro* storage at different temperatures (Chung *et al*., 1998).

Storage period (hours)	Sperm motility (%)			
	39°C	20°C	15°C	4°C
0	92.0±3.1	92.0±3.1	92.0±3.1	92.0±3.1
1	92.4±5.5 [a]	88.7±3.3 [a]	84.6±3.6[a]	77.2±4.0[b]
2	92.4±5.5[a]	81.9±3.9 [a]	76.9±4.8[a]	70.3±6.0[b]
4	84.3±13.3[a]	78.4±5.2[a]	68.7±5.9[b]	57.9±4.7[b]
8	62.7±10.3 [a]	70.0±4.3 [b]	62.5±5.8 [a]	53.6± 6.6 [a]
12	34.2±4.4 [a]	60.7±6.3 [b]	55.6±7.1 [b]	44.8±7.9 [a]
24	16.9±4.4 [a]	60.7±6.3 [b]	41.7±5.2 [b]	36.9±6.8 [b]
36	25.4±2.9 [a]	33.4±3.6 [b]	29.1±4.9 [b]	23.4±7.4 [a]
48	25.4±2.9 [b]	25.5±5.4 [b]	22.0±4.6 [b]	12.1±3.6 [a]

[a,b] Values within rows followed by different superscripts differ significantly ($p \leq 0.05$)

2.3 SEMEN EXTENDERS

The success of AI depends on the extent to which semen can be preserved without loss of fertility (Anil *et al*., 2004). Fresh semen is perishable and the way to improve storage life of fresh semen is dilution in an appropriate extender and storage at a constant cool temperature of 16–18 ^{0}C Laforest & Allard (1996). The semen extender enables the semen to remain in a viable state for a variable period without any loss in the fertilizing capacity of spermatozoa. Semen extender also supports AI with a minimum dose at minimal cost and health risk (Reed, 1990). Semen extenders lengthen the viability of diluted semen by protecting it against cold shock. Semen extenders act as a buffer against low pH, provide proper osmotic pressure and electrolyte balance, inhibit bacterial growth and supply nutrients to the sperm, as well as extend the use of the ejaculate so that more sows/gilts can be inseminated per ejaculate. Commercial boar semen

extenders vary in composition and are classified as short-term (<3 days) and long-term (>3 days) extenders based on how long they can preserve liquid semen without considerable loss in fertility (Anil *et al*., 2004; Umesiobi *et al*., 2004).

Reduction in fertilizing integrity of stored semen is a gradual process (beginning at ejaculation) regardless of the extender used (Anil *et al*., 2004; Umesiobi *et al*., 1999, 2004). The fertility of stored boar semen depends mainly on initial semen quality, number of spermatozoa per insemination and type of extender (Waberski, 1990), semen storage time and conditions, and interval between insemination and ovulation (Waberski *et al*., 1994a; b), number of inseminations (Correa *et al*., 2002), the interval between weaning to first service and parity of the female. A long-term extender is generally preferred to a short-term extender because the former ensures longer shelf life and thereby reduces the need for frequent semen delivery.

The viability of boar spermatozoa requires reliable methods of semen extension (Waberski *et al*., 1994b). However, according to Umesiobi (2004, 2006a) and Umesiobi *et al*. (2004), the dilution of spermatozoa has been shown to be deleterious, possibly because of the inherent interference by the plasma (Umesiobi & Iloeje, 1999) or the dilution of protective substance in the seminal plasma, although no specific components were identified.

Different types of semen extenders exist amongst which are conventional extenders such as Cornell University extender (CUE), Beltsville thawing solution (BTS) and Adrohep. Some of the renowned local extenders include egg yolk, honey, coconut water and most recently Palm wine plus 'Nche' (*Saccoglotis gabonensis*) (Umesiobi, 2004). Some reports indicate that BTS had no negative effect on three days semen storage (Alexopoulos *et al*., 1996; Hofmo, 2000). However, it was reported that the maximum recommended storage time of semen is usually associated with breed of male animal (Umesiobi *et al*., 2004),

the dilution level and the number of spermatozoa per AI (Umesiobi *et al*., 1999; Johnson *et al*., 2000).

Alexopoulos *et al*. (1996) found that AI doses of 3.0×10^9 spermatozoa diluted in (BTS) could be stored for 72 hours without negative effect on fertility. In contrast Johnson *et al*. (2000) reported that the farrowing rate for the semen stored for 21-30 hours was superior to that of the semen stored for 69-78 hours. However it was noteworthy that Alexopoulos *et al*. (1996) used double insemination and crossbred boars while Johnson *et al*. (2000) used single insemination and purebred boars. In another study by Waberski *et al*. (1994b), fertilization rates and number of accessory spermatozoa decreased between semen stored for 0-24 and 48-72 hours in BTS, and between semen stored for 0-24 and 48-72 hours in Androhep producing better results. Significant differences in fertility between diluents were seen only when using semen stored for more than 96 hours, with semen extended in Androhep producing the higher results (Waberski *et al*., 1994b). No decrease in pregnancy rate was reported by Waberski *et al*. (1990). However, the fertilization rate dropped between day 1 and day 2, as did the number of accessory spermatozoa in the day 3 semen samples (Weberski *et al*., 1994). The reduction in fertilizing capacity varies with extender, and fertilization rate and the number of spermatozoa remained constant over 2 day period with Androhep medium while there was already a drop in BTS in semen between day 1 and day 2 (Weberski *et al*., 1994c).

Table 2.3 demonstrates motility % and normal apical ridge% of semen extended in Androhep and Beltsville Thawing Solution (BTS). The results show that there was no significant difference between semen extended in Androhep and (BTS) in group 1 and group 2 but the significant difference was found to be present in group 3 and group 4 in both semen extenders. In terms of Normal Apical Ridges significant differences was observed between 1, 3 and between 4 and 5. Significant differences were also found between groups 2, 4 and 5.

Table 2.3 Motility (%) and normal apical ridges (%) in stored liquid boar semen extended in Androhep or Beltsville Thawing Solution (BTS) medium, average of 18 pooled ejaculates from 3 boars (Waberski *et al*., 1994b).

Group semen	Motility Androhep	Motility BTS	Normal Apical Ridge % Androhep	Normal Apical Ridge % BTS
1	92.6 [a]	92.0 [a]	93.3 [a]	92.6 [a]
2	90.0 [a]	87.3[b]	92.4[b]	86.6[b]
3	82.4[a]	77.1[b]	89.0[b]	84.4[b]
4	77.9[a]	71.8[b]	78.1[b]	74.1[c]
5	73.0[a]	61.7[b]	76.1[c]	73.9[a]

[a,b,c] Values within columns followed by different superscripts differ significantly (p≤0.05)

Storage time in 5 semen groups; 1) 0 to 24, 2) 24 to 48, 3) 48 to 72, 3) 72 to 96 and 96 to 120 hours.

2.3.1 Conventional semen extenders

2.3.1.1 Beltsville thawing solution

The storage tolerance of spermatozoa, without noticeable decrease in quality, depends, among other factors, on the choice of extender (Levis, 2000). In some studies, the short-term extender Beltsville thawing solution (BTS) had no negative effect on *in vivo* fertility after storage for 3 days (Alexopoulos *et al*., 1996; Hofmo, 2000).The maximum recommended storage time of semen is associated with the dilution level and the number of spermatozoa in the artificial insemination (AI) dose (Levis, 2000). The threshold number of spermatozoa for optimum fertility depends upon the individual male and semen quality (Colenbrander *et al*., 2000). Alexopoulos *et al*. (1996) found that AI doses of 3.0 X 10^9 spermatozoa diluted in BTS could be stored for 72 h without any negative effect on fertility. In contrast Johnson *et al*. (2000), using the same dose and extender, reported that the farrowing rate for semen stored for 21–30 h was superior to that of semen stored for 69–78 h.

2.3.1.2 Adrohep semen extender

Androhep was used in cattle (Vishwanath & Shannon, 2000), sheep (Paulenz *et al*., 2002), pig (Zou & Yang, 2000), canines (Igeur-ouada & Verestegen, 2001), elephant (Asher *et al*., 2000), deer (Graham *et al*., 2004) and even Bactrian camels (Chen *et al*., 1990) to protect and maintain spermatozoa during processing and storage. According to Musa *et al*. (1992), Androhep has been used as short-term semen extender for ≥3 days whereas some authors have recommended the use of Androhep extender with lactose and egg yolk for preservation of liquid semen up to 36 hrs.

2.3.2 Local semen extenders

2.3.2.1 Egg yolk semen extender

Usually, freezing extenders for boar semen contain egg yolk plus other agents such as buffers, additives and cryoprotectants. The most frequently used cryoprotectant is glycerol, which has to be used at low concentrations (under 4%) due to its potential toxicity (Buhr, 1990). Egg yolk-based diluents provide adequate cryoprotection for the sperm of several mammalian species. Traditionally, egg yolk is been used as additive for the freeze preservation of spermatozoa because of its wide availability. Variations in the chemical composition of the egg yolk of different avian species appear to influence the protection afforded during cooling, freezing, and thawing (Santiago-Moreno *et al*., 2008). However, the chemical composition of egg yolks of different avian species varies, particularly in terms of the cholesterol, fatty acid and phospholipid contents (Surai *et al*., 1999) and this influences the protection they afford during cooling, freezing, and thawing. Egg yolk from other bird species has successfully been used as additive for the freeze preservation of sperm in some species, especially equids. There were also numerous reports that egg yolk from duck, quail or chicken has different components of fatty acid, phospholipids and

cholesterol, which resulted in different cryopreservative effects on sperm (Trimeche *et al*., 1997; Bathgate *et al*., 2006).

The effect of egg yolk concentration may vary depending on the buffer system used. As suggested in earlier work with other species, egg yolk protects the sperm cell from the damaging effects of low temperature (Garde *et al*., 2007). Several hypotheses have been presented to account for this protective action. Recent evidence supports the idea that egg yolk may be used as the binding of deleterious major seminal plasma proteins on the sperm surface that would cause lipid efflux (Manjunath *et al*., 2007).

In the study conducted by Garde *et al.* (2007), the author determined the effect of egg yolk, cryoprotectant and various sugars on semen cryopreservation in endangered Cuvier's gazelle. Among diluent components which included BTS, CUE and Tes-tris, the author concluded that the concentration of egg yolk may be important for gazelle sperm cryopreservation since it has been reported for Mohor gazelle spermatozoa that a concentration of 20% egg yolk in a Tes-Tris-based diluent leads to poorer cryosurvival than a concentration of 5% egg yolk which could be mainly attributed to the lowered rate of fructolysis when boar semen is extended in 20% of egg yolk (Holt *et al*., 1996), but the results in this study showed lower survival rate of spermatozoa extended in egg yolk. However an earlier study reported that a high concentration of egg yolk is detrimental to Mohor gazelle spermatozoa (Holt *et al.,* 1996). The present report extends the data, showing that different avian egg yolks in extender have different cryoprotective actions on bull sperm cryopreservation (Trimeche *et al*. 1997) and showed a beneficial effect of quail yolk compared to chicken yolk in the Jackass, using 10% in extender.

2.3.2.2 Coconut water semen extender

There is a considerable interest inbiological alternatives like semen freezing using extenders that prolong sperm cell life such as coconut water extender, are cheap, easily prepared and raw materials (coconut) are abundant in some regions like Northeast Brazil (Rota 1998). These may help preserve and increase canids with valuable characteristics and those that are threatened by extinction. Coconut water extender with 7% glycerol was used successfully in cryopreservation of caprine semen and is often used in goat breeding as well as in pigs (Rota, 1998). Currently, there is a considerable interest in using coconut water solution (CWS) in studies on *in vitro* culture of oocytes (Blume *et al*., 1997) and embryos as well as for preservation of preantral follicles (Silva *et al*., 2000) and semen (Cardoso *et al*., 2002). The presence of supplements in the culture medium such as insulin-transferrin-selenium (ITS), pyruvate, glutamine, hypoxanthine, and bovine serum albumin (BSA) has improved the growth and survival of isolated bovine preantral follicles after *in vitro* culture (Katska *et al*., 1996). According to Blume *et al*. (1997), coconut water is obtained from green coconuts and filtered twice using paper filters. A stock solution is prepared containing 50% coconut water, 25% ultra-pure water and 25% of a 5% anhydrous monosodium citrate solution.

2.3.2.3 Palmwine plus 'Nche' (*Saccoglotis gabonensis*) Urban

Saccoglotis gabonensis urban is a tree commonly found in most parts of Nigeria, especially the south-eastern region (Umesiobi, 2004). Nigerian local palmwine tappers use the bark of Saccoglotis *gabonensis* urban to preserve palmwine against microbial degradation and possible extension of shelf life of palmwine (Umesiobi, 2004). The inability to store boar semen extended in a liquid state for several days without a significant reduction in fertility (Umesiobi, 2000a; Umesiobi *et al*., 2000) severely limits the utilisation of artificial insemination in pigs. Some of the factors responsible for lowered fertility of stored boar semen

are the rate of fructolysis (Umesiobi, 2000a; Umesiobi *et al*., 2000), poor semen extenders (Lai *et al*., 1993) reduced survival time of spermatozoa in the uterus, uterotubal junction and oviducts compared to survival of fresh spermatozoa (Umesiobi, 2000b). In the same study conducted by Umesiobi (2004), where the author compared the functional integrity of boar spermatozoa and sow fertility using local semen extender (Raphia *Raphia hookeri* Palmwine plus Nche *Saccoglottis gabonensis)* urban extender and compared it with Cornel University extender sperm motility, live sperm per cent, sperm concentration were highest in raphia palmwine + *S. gabonensis* extender followed by Cornel University extender, raphia palmwine extender. This could be an indication that "Umqombothi" extenders might also give positive results from the proposed study.

2.3.2.4 "Umqombothi" (*Sorghum bicolour*) semen extender

No confirmatory study on the use of "Umqombothi" as a local semen extender has been done yet. Numerous sorghum species are used for food, fodder, and the production of alcoholic beverages.

Figure 2.2 *Sorghum bicolour variant (Coutersy of Mothobi's Farm)*

Most species are drought tolerant and heat tolerant and are especially important in arid regions. They form an important component of pastures in many tropical

regions. Sorghum species are an important food crop in Africa, Central America and South Asia, and are the "fifth most important cereal crop grown in the world" (Umesiobi, 2000a).

Sorghum bicolour (Figure 2.2) is the primary sorghum species grown for grain for human consumption and for animal feed in most of the African countries. The species originated in northern Africa and can grow in arid soils and withstand prolonged droughts. It is commonly known simply as sorghum.

S. bicolour is usually an annual, but some cultivars are perennial. It grows in clumps which may reach over 4 meters high. The grain is small, reaching about 3 to 4 mm in diameter (see Figure 2.2). Sweet sorghums are sorghum cultivars that are primarily grown for foliage; they are shorter than those grown for grain. The species is a source of ethanol biofuel and in some environments may be better than maize or sugarcane as it can grow under more harsh conditions.

Uses and cultural aspects: *S. bicolour* is processed into a wide variety of nutritious traditional foods, such as semi-leavened bread, couscous, dumplings and fermented and non-fermented porridges. It is the grain of choice for brewing traditional African beers. New products, such as instant soft porridge and non-alcoholic malt beverages, are great successes. In the competitive environment of multinational enterprises, sorghum has been proven to be the best alternative to barley for lager beer brewing (Awika & Rooney. 2004).

2.3.2.4.1 Biochemical composition of sorghum

Sorghum bicolour is a cultivated, small-seeded tropical grass grown for food, feed or forage, providing the major source of dietary energy and protein for some one billion people in the semi-arid tropics (Belton & Taylor, 2004; Rooney, 2004) it is rich in vitamin B which normally has an alcohol content of approximately 3% when processed into home brew beer. According to Taylor *et al*. (1997),

unprocessed sorghum contains 11.6% protein, 3.8% fat and 16.0% fibre. *S. bicolour* is rich in phytochemicals known to significantly affect human health, such as tannins, phenolic acids, anthocyanins, phytosterols, and policosanols (Awika & Rooney, 2004). According to Awika & Rooney (2004), tannins of *S. bicolour* are almost exclusively of the ‘‘condensed’’ type. They are mainly polymerised products of flavan-3-ols and/or flavan-3, 4-diols. Glycosylated and non-glycosylated polymers of flavan- 4-ols with various substitution patterns have also been reported in sorghum. The phenolic acids of *S bicolour* largely exist as benzoic or cinnamic acid derivatives. As in other cereals, the *S. bicolour* phenolic acids are mostly concentrated in the bran (outer covering of grain). The phenolic acids exist mostly in bound forms (esterified to cell wall polymers) with ferulic acid being the most abundant bound phenolic acids in sorghum (Awika & Rooney, 2004). However, according to Demuyakor & Ohta (1993) polyphenols, mainly the PAs, have been reported to inhibit growth and fermentation of some micro-organisms and may influence the taste of the beer.

The study of phenolic compounds in beer is important as they are involved in flavour characteristics, foam maintenance, physical and chemical stability and shelf life of the beer (Montanari *et al*., 1999). The rich variety of non-volatile, low molecular weight phenolic compounds in sorghum may contribute to sensory properties. Both phenolic acids and polyphenols are present in beer, the majority originating from the malt (Montanari *et al*., 1999). Studies carried out to determine the effects of fermentation on the phenolic compounds of sorghum have mainly concentrated on the high molecular weight phenolic compounds (Obizoba & Atii, 1991; Nwanguma & Eze, 1996) though the low molecular weight phenolic compounds may have a role in the overall quality of the beer. Phenols help in the natural defence of plants against pests and diseases, while the plant sterols and policosanols are mostly components of wax and plant oils (Awika & Rooney, 2004). The phenols in sorghums fall under two major categories: phenolic acids and flavonoids. The phenolic acids are benzoic or cinnamic acid derivatives (Montanari *et al*., 1999), whereas the flavonoids include tannins and

anthocyanins as the most important constituents isolated from sorghum to date. The most common anthocyanins in sorghum are the 3-deoxyanthocyanidins and these anthocyanins have a small distribution in nature (Clifford, 2000) and are distinct from the more widely distributed anthocyanidins in that they lack a hydroxyl group at the C-3 position and exist in nature substantially as aglycones (Clifford, 2000). Sorghum phytosterols are similar in composition to those from maize and contain mostly free sterols or stanols and their fatty acid/ferulate esters (Avato *et al*., 1990; Singh *et al*., 2003). The sterols and stanols are structurally similar, except for the presence of a double bond at position 5 in sterols, which is lacking in stanols. The policosanols (fatty alcohols) exist mostly as free or esterified forms with C24–C34 atoms, and the general formula CH3–(CH2) n–CH2OH. Furthermore, sorghum has been shown to possess DPPH radical-scavenging activity and direct antimutagenic effects (Rooney, 2004). HMG-CoA reductase inhibitory activity has also been detected in methanol extracts of sorghum (Montanari *et al*., 1999). However, little information is available concerning the antimicrobial effects of sorghum.

High levels of p-coumaric acid have been observed in some white pericarp, non-tannin sorghums that are susceptible to moulding (Belton & Taylor, 2004). Phenolic acids increase during caryopsis development, reaching a maximum at physiological maturity and decreasing thereafter (Rooney, 2004). p-Coumaric acid is the progenitor of ferulic acid and its conversion might be deficient in susceptible cultivars. Sorghum cultivars resistant to fungal attack contained both a greater variety and higher amounts of free phenolic acids, especially in the case of tannin-containing sorghums (Rooney, 2004). The presence of a pigmented testa (Esele *et al*., 1993) as well as seed phenols and glume colour caused by phenolic pigments (Audilakshmi *et al*., 1999) also contribute to grain mould resistance. Funnell & Pedersen (2006) showed that both leaves and grain of sorghum bearing the gene for the brown midrib (bmr) trait are resistant to attack by various species of Fusarium. The bmr trait is a defect in the pathway of lignin biosynthesis and it has been proposed by Funnell & Pedersen (2006) that

lignin biosynthetic intermediates accumulating in the bmr lines contribute to the lowered growth of Fusarium.

2.3.2.4.2 Usefulness of sorghum bicolour as semen extender

Sorghum is a rich source of various phytochemicals including tannins, phenolic acids, anthocyanins, phytosterols and policosanols (Awika & Rooney, 2004) and these sorghum fractions possess high antioxidant activity *in vitro* relative to other cereals or fruits. However phenolic compounds affect the rate of fermentation, quality and stability of opaque beer produced from sorghum (Bvchora *et al*., 2004). The stages of traditional “Umqombothi” preparation involve the cooking of cereal meal, lactic acid fermentation, boiling of the lactic acid fermented mixture, first alcoholic fermentation, addition of the sweet, non-alcoholic beverage, straining, and the second alcoholic fermentation stages (Gadaga *et al*., 1999; Bvochora & Zvauya, 2001). The high energy content of *S. bicolour* might play a vital role in the process of fructolysis.

Chapter 3

Research design and methodology

3. METHODOLOGY

3.1 EXPERIMENTAL LOCATION AND ANIMAL MANAGEMENT

This study was conducted at the Germplasm Conservation and Reproduction Biotechnologies (GCRB) Unit of the Agricultural Research Council (ARC), Irene, South Africa 25° 52' 8" South 28° 12' 36" East and was aimed at determining improvements in the viability and fertilizing integrity of boar spermatozoa using the "Umqombothi" (*sorghum bicolour*) extender, during the period of November 2007 to May 2008. The experimental boars (n = 12) and sows (n = 12) were donated by the Nutrition Unit of ARC for the study. The 12 boars and 12 sows were randomly chosen for this experiment.

3.1.1 Weaners

The weaners were housed in the weaner house for a period of 4 weeks before moving to the grower house for random selection of sows. Piglets were introduced to a pelleted creep diet at the age of 2 weeks while still in the farrowing house; this was to ensure the diet adaptation by the piglets. This was followed by another type of pelleted feed mixed together with pelleted creep diet for a period of two weeks after which grower diet containing 18% crude protein and 14 MJ of DE/kg was fed for a week while pigs were still in the house. During the fourth week piglets were fed a grower diet. The diet was fed for a week after which piglets were moved to a grower house. Piglets occupied the weaner house for a period of 4 weeks.

3.1.2 Growing pigs

Pigs were moved from the weaner house to the grower house at the age of 9 weeks and at an average body weight of 23kg. Pigs were fed a grower diet *ad libitum.* Random selection of breeding sows in the grower house started from week 16 at an average weight of approximately 60kg. Selected sows were left in the grower house until they reached a weight of 120kg at the age of approximately 28 weeks and were taken to the sow house for breeding. Pigs occupied the grower house for 8 weeks.

3.1.3 Gilts

Upon arrival at the sow house, gilts were vaccinated against E. coli, parvovirus, Erysipelas and Leptospirosis, and were treated against parasites. Gilts were housed individually and fed 2kg of boar and sow diet (13% protein and 13 MJ DE per kg) in the sow house. Gilts were inseminated at the age of 28 weeks in the farrowing house. Two weeks before farrowing, gilts received a vaccination against E.coli.

3.1.4 Lactating sows

Dry sows were moved from the sow house about a week before farrowing. The purpose was to ensure that sows adapted to the surroundings before farrowing. At this stage the sows are fed 2kg of lactating sow meal per day. After farrowing sows were fed lactacting sow diet *ad libitum* 13% crude proteins and 14 MJ DE per kg. Sows occupied the farrowing house for 5 weeks: 1 week for adaptation and 4 weeks for lactation.

3.1.5 Boars and dry sows diet

Boars and dry sows were fed 2kg of boar and sow meal on a daily basis.

3.2 EXPERIMENTAL DESIGN

This study involved two distinct experiments aimed at determining the suitability of the use of “Umqombothi” *Sorghum bicolour* extender for the improvement of the viability and fertilizing integrity of boar spermatozoa. Experiment 1 evaluated the effects of different semen extenders being semen which was not diluted (UNX), semen extended in “Umqombothi”: (UMQ), and semen extended in Beltsville Thawing Solution (BTS) and storage temperature (4°C and 25°C) on the viability and fertilising capacity of boar spermatozoa. Experiment 2 dealt with the effects of time of semen collection (9:00 or 15:00) and semen extenders on semen quality.

These research protocols were conducted in a 2 x 3 factorial experiment. The overall experimental protocols lasted for 24 weeks.

3.3 BOAR TRAINING

Step 1: Training of the boar involved introducing the boar to the sow house, then moving it around the house for possible sexual stimulation.
Step 2: Sows on heat were serviced by the experienced boars in the presence of the trainee boar.
Step 3: The trainee boar was introduced to the sow to allow possible mounting.
Step 4: Trainee boars that did not want to mount sows on heat were introduced to the dummy sow which was smeared with the saliva, semen and urine of experienced boars.

3.4 SEMEN COLLECTION

Immediately after one week of training, semen was collected from each boar once a week into 50ml bottle per boar for three consecutive weeks. Semen was collected once a week at 9:00 or 15:00 from the experimental boars using a gloved-hand technique following the procedures of Umesiobi (2008b) and used for artificial insemination (AI) services. Semen from each boar was deposited into 50ml container immediately after it was harvested from the boars and sent to the (ARC) (GCRB) laboratory where semen was evaluated immediately on arrival at the temperature of about 28°C with the aid of water bath filled with warm water, also at approximately the same temperature to avoid heat or cold shock to the sperm cell.

3.5 PREPARATION AND TECHNIQUE FOR COLLECTION: GLOVED-HAND TECHNIQUE

3.5.1 A collection container was filled with warm water at about 37°C. The collection container contained a plastic sterile collection bag and the top of the container was covered with absorbent gauze to filter out the gel portion of the semen.

3.5.2 The boars were allowed to mount the dummy sow or a sow that was in oestrus.

3.5.3 The end of the penis was grabbed firmly with a gloved hand and the collection process was initiated with firm pressure to the spiral end of penis with the hand so that the penis could not rotate in the hand. This process imitated the pressure applied by the corkscrew shape of the sow's cervix.

3.6 "UMQOMBOTHI" PREPARATION

The ingredients used were: equal measures of (maize meal) crushed maize malt and crushed sorghum malt. The maize malt provided a lighter-toned beer with a mellower flavour. The sorghum malt provided a darker beer.

The ingredients were mixed in a cast-iron pot, known as a potjie in South Africa. Four measures of warm water were added. The mixture is left overnight. The mixture started fermenting while bubbles appear. A sour odour could be detected. A small portion of the maize-flavoured water was removed and put to one side. The remaining mixture was then cooked until crusty sediment formed. This product is known as isidudu and can be eaten as porridge. When making beer, the isidudu is left to cool for a day.

After the mixture cooled, it was poured into a large plastic vat. The liquid that was set aside was added to the vat. A handful of sorghum malt and a handful of maize malt were added to the vat. The brew was stirred with a traditional stirring spoon called an ipini. The vat was covered with a lid and blanket (to retain heat). The vat was put in a warm place overnight, to encourage fermentation.

The traditional method of testing to see if the brew is ready is to light a match close to the vat. If the match goes out quickly, the brew is ready. If the match remains lit, the brew is not ready. This is because the fermenting mixture is producing large amounts of carbon dioxide, which does not allow for combustion of the match.

When the brew is ready, the mixture is filtered through a large metal strainer, to collect the excess maize. The sediment at the bottom of the vat is known as intshela. The intshela is added to the filtered beer, to give extra flavour.

The maize solids, collected from filtering, are squeezed out. This maize is usually cast onto the ground for chickens. The brewer of the beer traditionally gives thanks to the ancestors while casting the maize.

3.7 SEMEN EXTENSION

In treatment 1, semen was collected at 9:00, divided into three treatment groups and thereafter extended with either ”Umqombothi” (UMQ) or Beltsville Thawing Solution (BTS), and the control which was unextended semen (UNX) per ejaculate and stored at 4°C and 25°C, respectively. The semen samples from each of the three treatment groups were stored and their viability evaluated after three days of storage. In treatment 2, semen was collected at 9:00 or 15:00 time interval, the samples were then extended with either (UMQ) or (BTS), and the control which was unextended semen (UNX). Semen viability from the three treatment combinations were determined immediately from both extended and unextended samples.

Semen quantity and quality for AI are currently assessed according to four basic traits (semen volume, sperm concentration, progressive motion of spermatozoa also known as sperm motility and proportion of abnormal spermatozoa) whose values can often be difficult to interpret. All these traits are only partial semen characteristics as it is impossible to determine semen quality on the basis of only one of these traits without taking into account the remaining ones; therefore it is desirable to combine the traits mentioned above to an overall characteristic, e.g. number of viable spermatozoa (Smital *et al*., 2004). Immediately after collection, semen was taken and evaluated in ARC (Irene) (GCRB) laboratory whereby semen was evaluated for volume, pH using a pH meter (827 pH lab Metrohm), concentration using a spermacue, motility, survivability, and morphology using a microscope, and a warm plate used for warming up the slides and the covers. A regulated vacuum pump was used to filter the “Umqombothi.

3.8 SEMEN EVALUATION

Following extension, semen samples from the three treatment groups were sent to the (GCRB) laboratory where they were evaluated for volume, sperm concentration, motility, abnormal sperm and semen pH. Semen volume was determined using twelve 50 ml sterilized bottles which indicated in the measurements of each sample of semen harvested from each of the twelve selected large white boars. Sperm concentration was evaluated by spermacue (Minitube) an instrument in which sample is inserted into and gives the reading of the sperm concentration on the screen. The samples were inserted into the spermaque using a plastic pipette and this determined the concentration by giving the readings on the displaying screen (see Plate 3.3). Sperm morphology was evaluated by using a phase contrast microscope (see Plate 3.4). Before the evaluation of sperm morphology started, 12 slide covers were placed on the slide warmer at 37°C which sustained the spermatozoa temperature so that the motility percentage of sperm could not be affected by the temperature fluctuations before the evaluation. Under the phase contrast microscope the examination involved a thin smear of semen stained with an eosin/nigrosin stain, which can also be used to determine the percentage of live/dead sperm. It determined the percentage of abnormal sperm (head, body and tail defects). These may account for 0 – 20% of total count in a normal semen sample. Semen samples from 4 boars were placed on the slides and evaluated under the microscope, the readings were given with scores between 80 and 0. Sperm motility was evaluated simultaneously with sperm morphology using the same procedures but in this instance it did not include the staining of semen. Semen pH was evaluated by means of a pH meter (827 pH lab Metrohm) (see Plate 3.5), the semen samples from the 4 boars were drawn from the beaker with a plastic pipette and inserted into the pH meter which gave the reading of the each sample's pH.

3.8.1 Sperm concentration

Sperm concentration was evaluated by the use of a spermacue (Minitube). This equipment was used for all unextended (UNX) semen as well as samples of semen extended in BTS and UMQ. Semen samples were inserted into the spermacue and the concentration readings were derived from each of the boar semen samples inserted.

***Plate 3.1**. Spermacue (Minitube). Courtesy of ARC (Irene)*

3.8.2 Sperm motility

Immediately after fresh semen was collected from the boars, a phase contrast microscope was used to evaluate sperm motility, live sperm percentage and acrosome intergrity of all unextended (UNX) semen samples. Sperm motility was also determined using aliquots of semen from the BTS and UMQ treatment groups.

***Plate 3.2** The Phase contrast microscope. Courtesy of ARC (Irene)*

3.8.3 Semen pH

Freshly harvested semen pH was measured with the aid of a pH meter (827 pH lab Metrohom) as well as that of semen extended in BTS and UMQ.

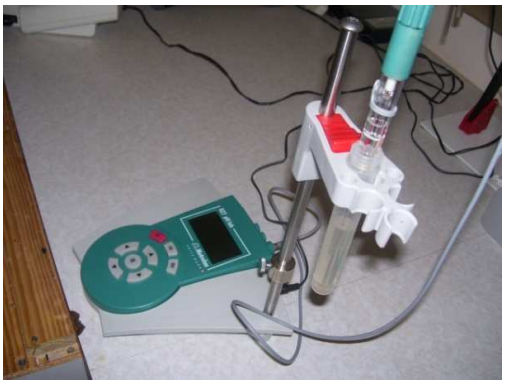


Plate 3.3. *pH meter (827 pH lab Metrohm).*

3.8.4 Semen volume

Twelve 50ml sterilised bottles (Schott®) indicating the volume were used to measure each sample of semen harvested from each of the twelve selected Large White boars.

3.9 SOW OESTRUS INDUCTION PROCEDURE AND ARTIFICIAL INSEMINATION

3.9.1 Oestrus induction in sows

Oestrus was synchronised in the experimental sows by a single subcutaneous injection of P.G. 600® (400 IU PMSG with 200 IU HCG/5 mL dose/animal; Intervet Inc., Millsboro, DE). Sows were checked for oestrus twice daily by providing them with fence-line contact with a mature boar, for a minimum of 15 minutes beginning 12 hours after the injection of PG 600®. About 72 hours after

the PG 600® injection, all sows were given 1000 IU of HCG (Intervet Inc., Millsboro, DE), to induce ovulation to occur at 40 hours (Umesiobi *et al*., 2002; Willenburg *et al*., 2003; Umesiobi, 2006b). After the onset of oestrus, sows on each treatment were artificially inseminated using semen from the same boars and collections.

3.9.2 Artificial insemination

All experimental females received inseminations of 3 x 10^9 sperm/80 ml at 12 and 24 hours after onset of oestrus following the recommendations of Umesiobi (2006a) and Umesiobi *et al*. (2002). All females were inseminated using a spirette catheter® (Minitube Inc., Verona, WI). Fertility was exemplified by conception rate defined as non-return rate (NRR), farrowing rate and litter size (based on total as well as live pinglets) were recorded. After semen evaluation, the remaining semen samples from the respective treatment groups were used to inseminate the experimental sows.

3.10 STATISTICAL ANALYSES

This study involved a 2 x 3 factorial experiment. Data on semen viability were analysed using the general linear model procedure of Statistical Analysis System, Version 9.1 (SAS, 2002). The statistical model included semen viability classification of unextended semen (UNX), semen extended in BTS and semen extended in “Umqombothi” (UMQ) within treatment groups. All the fertility data were analysed for Analysis of Variance (ANOVA) using a Completely Randomised Design (CRD) (SAS, 2002).

Chapter 4

Results and discussion

4.1 RESULTS

4.1.1 Experiment 1: The effects of semen extenders and storage temperatures on the viability and fertilising capacity of boar spermatozoa

In this experiment semen samples in the various experimental groups (unextended semen (UNX), ”Umqombothi” (UMQ) and Beltsville Thawing Solution (BTS) extenders) were stored at either 4^{o}, or 25^{o}C for 3 days, respectively. The effects of semem extenders on the viability of boar spermatozoa are presented in Table 4.1. In this study, there was a significant difference in sperm motility between unextended semen, ”Umqombothi” (UMQ) and Beltsville Thawing Solution (BTS) extenders, with (UMQ) exhibiting the highest percentage of motile spermatozoa, followed by (BTS). The UNX produced the lowest percentage of motile sperm cells (Table 4.1). The results show that there was no significant difference in live sperm percentages between (UNX) and (UMQ), but (UNX) and (UMQ) differed significantly from (BTS) which had the highest percentage of live sperm. Nevertheless no significant difference was observed in sperm concentration and semen pH between (UNX), (UMQ) and (BTS)

Table 4.1 Effects of semen extenders on boar semen viability

Parameters	UNX	UMQ	BTS	F-probability	SEM
Sperm motility (%)	51.2[a]	70.0[b]	66.2[c]	0.001	7.06
Live sperm (%)	72.5[a]	71.2[a]	87.5[b]	0.008	2.60
Spermcon ($x10^6$/ml)	190.8[a]	192.2[a]	193.8[a]	0.969	83.37
Semen pH	7.400[a]	7.415[a]	7.402[a]	0.992	0.0909

[a,b,c] Values with different superscripts within the row differ significantly ($p<0.05$)
UNX= Unextended semen
UMQ="Umqombothi"
BTS= Beltsville Thawing Solution

As indicated in Table 4.2, significant improvements in sperm motility, live sperm percentage and sperm concentration per ml were obtained from semen that were stored at 4°C. However no significant difference in semen pH was found when semen was stored at either 4° or 25°C, however 25°C had a higher value of motility percentage and live sperm was better at 4°C.

Table 4.2 Effects of storage temperature on boar sperm viability

Parameters	4°C	25°C	F-probability	SEM
Sperm motility (%)	41.2[a]	51.2[b]	0.001	2.04
Live sperm (%)	75.0[a]	71.2[b]	0.001	1.614
Sperm con. ($x10^6$/ml)	190.8[a]	199.0[a]	0.339	11.14
Semen pH	7.4[a]	7.5[a]	0.573	0.0315

[a,b] Values with different superscripts within the row differ significantly ($p<0.05$)
UNX= Unextended semen
UMQ="Umqombothi"
BTS= Beltsville Thawing Solution

The interaction effects of semen extenders and storage temperatures on the viability of boar sperm cells are depicted in Table 4.3. In this study, semen extenders in conjunction with storage temperatures exerted significant effects ($p<0.05$) on the sperm parameters. Highest sperm motility values were obtained from sperm samples extended in UMQ at 4°C. The lowest sperm motility was recorded in UNX at 4°C. In the same manner, the highest percentage of live sperm (77.5 ± 2.60) were observed in UMQ at 4°C, while as the poorest percentage live sperm cells (45.5 ± 2.60) were noted in UNX stored at 4°C. Highest sperm concentration per ml (202.5 ± 8.37) were recorded from the BTS semen samples that were stored at 4°C, with the lowest percentage of abnormal spermatozoa were obtained the BTS experimental samples at 4°C. On the contrary, no significant differences existed in semen pH between UNX, UMQ and BTS semen samples, and between the two storage temperatures.

Table 4.3 Interaction effects of semen extenders and storage temperatures on boar sperm viability

Parameters	UNX		UMQ		BTS		SEM
	25°C	4°C	25°C	4°C	25°C	4°C	
Sperm motility (%)	51.2^{a}	49.2^{a}	65.0^{b}	72.50^{b}	53.8^{c}	66.2^{d}	2.39
Live sperm (%)	48.8^{a}	45.5^{a}	71.2^{b}	77.5^{c}	71.2^{b}	67.5^{d}	2.60
Sperm con. ($x10^{6}$/ml)	187.9^{a}	190.8^{b}	184.5^{a}	193.0^{b}	194.5^{a}	202.5^{c}	8.37
Abnormal sperm (%)	10.0^{a}	10.0^{a}	12.5^{b}	11.2^{b}	7.5^{c}	2.5^{d}	1.021
Semen pH	$7.\ 4^{a}$	7.4^{a}	7.4^{a}	7.5^{a}	7.5^{a}	7.4^{a}	0.090

^{a,b,c,d}Values with different superscripts within the row differ significantly ($p<0.05$)
UNX= Unextended semen
UMQ="Umqombothi"
BTS= Beltsville Thawing Solution

As elucidated in Table 4.4, the two storage temperatures (25°C and 4°C) significantly ($p<0.05$) influenced the fertility rate in the experimental sows. Significant differences were noted in non-return rate, farrowing rate, total piglets and live piglets between semen stored at 25°C and 4°C. Higher values in non-return rate, farrowing rate, total piglets and live piglets were recorded from semen samples stored at 4°C.

Table 4.4 Effects of semen extenders on fertility rate in artificially inseminated sows

Parameters	25°C	4°C	SEM
Non-return rate (%)	75.3[a]	88.2[b]	1.614
Farrowing rate	70.25[a]	86.41[b]	2.60
Total piglets	6.2[a]	8.32[b]	1.89
Live piglets	3.0[a]	5.60[b]	1.13

[a,b]Values with different superscripts within the row differ significantly ($p<0.05$)

There was a significant difference in non-return rate between UNX semen samples stored at 25°C or 4°C, with the highest percentage of non-return rate found in those samples that were stored at 4°C (Table 4.5). Also, significant differences in farrowing rate, total piglets and live piglets were observed between the two storage temperatures. The highest values in Non-return, farrowing rates, total piglets and live piglets were obtained from BTS at a lower temperature of 4°C.

Table 4.5 Interaction effects of semen extenders and storage temperatures on fertility rate in artificially inseminated sows

Parameters	UNX		UMQ		BTS		SEM
	25°C	4°C	25°C	4°C	25°C	4°C	
Non-return rate (%)	59. 1^{a}	72. 5^{b}	68.2^{c}	88.36^{d}	66.4^{c}	89. 3^{d}	2.89
Farrowing rate	53.5^{a}	52.8^{a}	65. 3^{b}	82. 7^{c}	67.4^{b}	88.2^{d}	2.040
Total piglets	4.2^{a}	4.9^{a}	6.2^{b}	8.5^{c}	6.6^{d}	8.8^{d}	5.00
Live piglets	3.4^{a}	3.2^{a}	4.1^{b}	7.9^{c}	6.2^{d}	8.4^{d}	3.95

^{a,b,c,d} Values with different superscripts within the row differ significantly ($p<0.05$)

4.1.2 Experiment 2: The effects of time of semen collection and extenders on viability and fertilising capacity of boar spermatozoa

As indicated in Table 4.6 time (9:00 or 15:00) of semen collection had no significant effects on sperm motility percentage, live sperm percentage, sperm concentration, abnormal sperm percentage. However, higher percentage of live sperm cells was recorded from semen samples that were collected in the morning (9:00) hours; although, about 193.8 x 10^{6} of sperm concentration per ml was obtained during the afternoon (15:00) period.

Table 4.6 Effects of time of semen collection on boar semen viability

Parameters	9:00	15:00	F-probability	SEM
Sperm motility (%)	65.0	60.0	0.078	1.67
Live sperm (%)	80.7	74.2	0.100	2.12
Sperm con. (x10^{6}/ml)	190.7	193.8	0.754	6.83
Semen pH	7.3	7.5	0.315	0.0742

^{a,b} Values with different superscripts within the row differ significantly ($p<0.05$)
UNX= Unextended semen
UMQ="Umqombothi"
BTS= Beltsville Thawing Solution

The results from table 4.7 show that the UNX semen samples collected at 9:00 and 15:00 were significantly different in sperm motility live, sperm percentage, sperm concentration and abnormal sperm, with no significant differences in the semen pH values. The results also showed significant difference between UNX, UMQ and BTS) semen samples in all semen viability parameters, except the pH which was not affected by the experimental treatment combinations. Although highest percentage live sperm cells (80.0 ± 6.12%) were obtained from UMQ semen samples collected at 9:00, the highest sperm concentration per ml (207.5 ± 8.37 x 10^6) was observed in the UMQ semen that were collected at 9:00.

Table 4.7 Interaction effects of time of semen collection and semen extenders on boar semen viability

Parameters	UNX		UMQ		BTS		SEM
	9:00	15:00	9:00	15:00	9:00	15:00	
Sperm motility (%)	55.0^a	47.5^a	67.5^b	72.5^b	72.5^b	60.0^c	2.04
Live sperm (%)	47.5^a	50.0^a	80.0^b	75.0^b	70.0^c	65.0^c	6.12
Sperm con. (x10^6/ml)	193.5^a	188.0^b	207.5^a	198.5^b	186.0^c	180.0^d	8.37
Abnormal sperm (%)	10.5^a	17.5^b	5.2^a	11.5^c	5.0^a	10.0^c	1.021
Semen pH	7.3^a	7.5^a	7.4^a	7.3^a	7.3^a	7.5^a	0.090

^{a,b,c,d} Values with different superscripts within the row differ significantly ($p<0.05$)
UNX= Unextended semen
UMQ="Umqombothi"
BTS= Beltsville Thawing Solution

Semen parameters exemplified by non-return rate, farrowing rate, total piglets where not influenced by time of semen collection (Table 4.8).

Table 4.8 Effects of time of semen collection on fertility rate in artificially inseminated sows

Parameters	9:00	15:00	F-probability	SEM
Non-return rate (%)	88.3	87.9	0.100	2.12
Farrowing rate	82.6	83.4	0.697	2.89
Total piglets	8.3	8.3	0.670	1.318
Live piglets	6.2	5.9	0.612	2.20

Within the UNX, UMQ and BTS semen samples collected at 9:00 or 15:00, there was no significant difference ($p>0.05$) in no-return rate percentage, farrowing rate, total piglets and live piglets. However, significant changes were noted between treatment groups at various collection periods in non-return rate percentage, farrowing rate, total piglets and live piglets, with the highest farrowing rate and total piglets obtained from the BTS semen samples that were collected in the morning (9:00) hours. The UNX treatment group had the lowest percentage of non-return rate, farrowing rate, total piglets and live piglets at the various periods of semen collection

Table 4.9 Interaction effects of semen extenders and time of semen collection on fertility rate in artificially inseminated sows

Parameters	UNX		UMQ		BTS		SEM
	9:00	15:00	9:00	15:00	9:00	15:00	
Non-return rate (%)	71.3^{a}	72.3^{a}	86.36^{b}	85.99^{b}	87.3^{b}	86.9^{b}	4.21
Farrowing rate	54.8^{a}	55.1^{a}	85.3^{b}	83.0^{b}	84.9^{b}	82.2^{b}	4.56
Total piglets	4.8^{a}	4.9^{a}	7.0^{b}	6.9^{b}	8.01^{c}	7.9^{c}	6.69
Live piglets	3.5^{a}	3.6^{a}	6.0^{b}	5.8^{b}	6.1^{b}	5.9^{b}	2.89

^{a,b,c} Values with different superscripts within the row differ significantly ($p<0.05$)
UNX= Unextended semen
UMQ="Umqombothi"
BTS= Beltsville Thawing Solution

4.2 DISCUSSION

The success of AI depends on the extent to which semen can be preserved without loss of fertility (Anil *et al*., 2004). The semen extender enables the semen to remain in a viable state for a variable period without any loss in the fertilizing capacity of spermatozoa. Fresh semen is perishable and the way to improve storage life of fresh semen is dilution in an appropriate extender and storage at a constant cool temperature (Laforest & Allard (1996). Semen extender also supports AI with a minimum dose at minimal cost and health risk (Reed, 1990). Semen extenders lengthen the viability of undiluted semen by protecting it against cold shock. Semen extenders also act as a buffer against low pH, provide proper osmotic pressure and electrolyte balance, inhibit bacterial growth and supply nutrients to the sperm, as well as extend the use of the ejaculate so that more sows/gilts can be inseminated per ejaculate. Moreover, the viability of boar spermatozoa requires collecting semen within a period that has conducive climatic conditions (Umesiobi, 2001).

In this study, semen extenders (”Umqombothi” (UMQ) and Beltsville Thawing Solution (BTS)), time (9:00 and 15:00) of semen collection and storage temperature (25^{o} and 4^{o}C) exhibited significant effects on sperm quality and fertility rates in sows. The highest motile sperm cell and live sperm percentages and sperm concentration per ml were obtained from the UMQ treatment group, mostly from semen samples that were stored at ^{4oC}. However, the BTS semen samples ranked second in exhibiting improved sperm viability. The lowest sperm viability values were obtained from the UNX control group. These findings are in agreement with Laforest and Allard (1996) and Umesiobi (2000a, b), who noted that fresh and unextended semen is perishable and might lower the fertility and viability of boar semen and the way to improve storage life of fresh semen is by diluting semen in an appropriate extender and storage at a constant cool temperature of 16–18 ^{0}C. The objective of estimating sperm motility is to determine the motile proportion of spermatozoa and the proportion moving

progressively, i.e., actively moving forward. The results also showed that there was no significant difference in live sperm percentages between UNX and UMQ, however BTS differed significantly ($p<0.05$) between UNX and UMQ in percentage live sperm. In this regard, Van Groenland (1993) and Umesiobi (2004) stated that sperm motility extended in most conventional extenders such as the BTS only starts to decrease 24 hours. Nevertheless no significant difference was observed in sperm concentration and semen pH between UNX, UMQ and BTS.

The results from the study also showed that sperm motility and live sperm percentage of semen stored at 4°C differed significantly from those stored at 25°C, with higher values recorded from samples at 25°C. This is in compliance with the study by Haung *et al*. (2004) who concluded that ejaculated boar spermatozoa are vulnerable to cold shock and prolonged storage of boar spermatozoa at low temperatures reduces survival rate resulting in bottlenecks for the extension of artificial insemination in pig husbandry and Parks *et al*. (1992) observed that boar sperm motility remain active at 22°C than at higher temperatures. Umesiobi (2004) reported that local semen extenders, such as *Raphia hookeri* palmwine plus 'Nche' (*Saccoglotis gabonensis*) have cushioning effects on sperm quality under prolonged periods of semen storage. However (Kommisrud *et al* 2002) concluded that transfer of sperm cells from seminal plasma to artificial media has shown to decrease sperm viability particularly unextended semen and increase sperm agglutination. On the other hand, Dumpala (2007) stated that boar semen is extremely sensitive to sudden chilling and thawing procedures and require particular care during processing.

A previous study by Rozeboom (2001) showed that the fertility potential of extended porcine semen stored at 15°C yields acceptable fertilization rates, while exposure to temperatures below 15°C would result in cold-shock and cell death, the same results were found by (Lai *et al*., 1993) were the authors concluded that

fertility of liquid semen is gradually lost during extended periods of ambient temperatures.

A significant difference in abnormal sperm percentage was observed between UNX, UMQ and BTS and UMQ had the lowest percentage of abnormal sperm percentage followed by BTS. The lowest percentage of abnormal sperm was recorded from UNX, mostly at 25^{o}C of storage. This observation is in line with the study by Borg et al. (1993), who indicated that dilution of boar spermatozoa reduces protein and natural anti-oxidants along with other components in the seminal plasma, which are required for normal functioning and membrane integrity of the sperm. However (Juonala *at al*., 1998) found a positive correlation between many semen evaluation parameters, whereby loose abnormal heads,

The results showed that semen stored at 4°C had a higher percentage of non-return rate, farrowing rate, total piglets and live piglets. However, this result is in disagreement with the study by Santiago-Moreno *et al*. (2008) who reported that storage of boar semen at 4-8°C causes lower conception rate and embryo survival and attributed it to lower percentages of non-return rate, farrowing rate, total piglets and live piglets, compared with fresh semen. Farrowing failure is assumed to be the inverse of farrowing rate. Santiago-Moreno *et al*. (2008) argued that the likelihood of farrowing failure in sows increased with ageing of semen. However (Juonala *et al*., 1998) found that litter size should not be used for semen evaluation studies because there are many more important factors that influence litter size such as age, breed, health and nutritional state of the gilts, the number of gilts in oestrus, genetic factors, amongst others.

Time of semen collection (9:00 or 15:00) did not exhibit any significant effect ($p>0.05$) on boar semen viability. However, interactions between time of semen collection and semen extenders produced significant effects ($p<0.05$) on al the sperm quality parameters, except semen pH was not significantly affected by the treatment combinations. Further, time of semen collection did not affect fertility

rate in the AI sows. Nevertheless, interactions between time of semen collection and semen extenders had significant effects on non-return and farrowing rates, total piglets and live piglets. According to Rothschild (1996), differences in boar fertility are mainly due to genetics and not only due to environmental effects. The detection of these differences in boar fertility is very important for pig producers because the impact of males on herd reproduction performance is high, particularly when AI is used (Juonala *et al*., 1998). In the study conducted by (Anil *et al*., 2006) it was noted that commercial boar semen extenders vary in composition and are classified as short-term (<3 days) and long-term (>3 days) extenders based on how long they can preserve liquid semen without considerable loss in its fertility. The reduction in fertility of stored semen is a gradual process, beginning at ejaculation, regardless of the type of the extender used. Anil *et al*. (2004) stated that long-term extenders are generally preferred to a short-term extender because the former ensures longer shelf life and thereby reduces the need for frequent semen delivery.

Chapter 5

CONCLUSION AND RECOMMENDATIONS

5.1 GENERAL CONCLUSION

It can therefore, be concluded that UMQ produced the best results in sperm viability and fertility rate, mostly from semen samples that were collect during the morning hours (9:00) and stored at 4^{o}C. The results of this study demonstrate that semen extended in ”Umqombothi” (UMQ) is most suitable to be used in AI services. This suggestion rests on the observation that semen in UMQ produced the best semen quality and fertility rates in AI sows.

The success of AI depends on the extent to which semen can be preserved without loss of fertility. UMQ enables semen to remain in a viable state for about 3 days without a loss in the fertilizing capacity of spermatozoa and thus it was conveniently use in AI services with a minimum dose at minimal cost. It can also be concluded that storage temperature of boar semen has an effect on the viability of boar spermatozoa. On the other hand, as portrayed in this study, time of semen collection cannot be used as a criterion for the evaluation of boar semen viability. This is mostly because, from this study it was observed that time of semen collection did not have an effect on boar semen viability and fertility. Nevertheless were semen extenders were compared (UNX) collected at 9:00 and 15:00 differed significantly from UMQ and BTS collected at 9:00 and 15:00 whereby UNX had the lowest percentage of non-return rate, farrowing rate, total piglets and live piglets.

5.2 RECOMMENDATIONS

Results from this study suggest that UMQ can be used as short term semen extender at the storage temperature of 4°C. The success of AI depends on the extent to which semen can be preserved without loss of fertility. Therefore, UMQ enabled semen to remain in a viable state for 3 days without a loss in the fertilizing capacity of spermatozoa. Lowest percentages of abnormal sperm were obtained when “Umqombothi” was used to extend the semen samples. However litter size should not be used as for semen evaluation studies because there are many factors that influence litter size such as age, breed, health and nutritional state of the gilts, number of gilts in oestrus, genetical factors, amongst others. Further studies are needed to confirm the interaction effects between time of semen collection and semen extenders on boar semen viability and sow fertility.

REFERENCES

Ahmadi, A. & Ng, S.C. 1999. Fertilizing ability of DNA-damaged spermatozoa. J Exp. Zool. 284: 696–704.

Alexopoulos, C., Boscos, C., Saratsis, P., Saalidis, C. & Kryakiss, S. 1996. The effects of storage time and number of spermatozoa per insemination dose in semen characteristics and fertilizing capacity of boar semen diluted with Beltsville thawing solution (BTS) extenders. Anim Sci. 62: 599-604.

Alvarez, J.G. & Storey, B.T. 1983. Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. Biol. Reprod. 29: 548-555.

Alvarez, J.G. & Storey, B.T. 1992. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. J. Androl. 13: 232–241.

Anil, S.S., Larriestra, A., Deen. J., Morrison, R.B. & Miion, L. 2004. A retrospective study on the preserving capacity of a commercial boar semen extender. Theriogenol. 62: 425–436.

Asher, G.W., Berg, D.K. & Evans, G. 2000. Storage of semen and artificial insemination in deer. Anim. Reprod. Sci. 62: 195–211.

Audilakshmi, S., Stenhouse, J.W. & Reddy, T.P. 1999. Genetic analysis of grain mould resistance in coloured sorghum genotypes. Euphytica 116: 95–103.

Avato, P., Bianchi, G. & Murelli, C. 1990. Aliphatic and cyclic lipid components of Sorghum plant organs. Phytochem. 29: 1073–1078.

Awika, J.M., & Rooney, L.W. 2004. Sorghum phytochemical and their potential impact on human health. Phytochem. 65 : 1199–1221.

Baltàì Moner, J. 1996. La ventilacio´n por sobrepresio´n en las explotaciones porcinas. Nuestra Caban˜a 270 : 64–68.

Baltàì Moner, J. 1997. La ventilacio´n positiva, una te´cnica para optimizar las condiciones ambientales de la maternidad ydestete en las explotaciones de porcino. *Produccio´n Anim*. 120: 25–32.

Barth, A.D. 1992. The relationship between sperm abnormalities and fertility. In: Proc 14th Tech Conf Artif Insem Reprod Nat Assoc Anim Breeders, 47-63.

Bathgate, R., Maxwell, W.M. & Evans, G. 2006. Studies on the effect of supplementing boar semen cryopreservation media with different avian egg yolk types on *in vitro* post-thaw sperm quality. Reprod. Domest. Anim. 41: 68–73.

Belton, P.S. & Taylor, J.R.N. 2004. Sorghum and millets: protein sources for Africa. Trends in Food Sci. Technol. 15: 94–98.

Blume, H., Vale Filho, V.R., Marques, A.P., Saturnino, H.M. 1997. Avaliação da água de coco na maturação de oócitos bovinos. Rev. Bras. Reprod. Anim. 21: 72–75.

Borg, K.E., Lunstra, D.D. & Christenson, R.K. 1993. Semen characteristics, testicular size and reproductive hormone concentration in mature duroc, meishan, fengiing and minzhu boars. Biol Reprod. 49: 515-21.

Braundmeier, A.G., Shanks, R.D. & Miller, D.J. 2002. The relationship of porcine zona binding ability to fertility. Biol. Reprod. 66(Suppl. 1): 274–275.

Buhr, M.M. 1990. Preservation of boar sperm alters membrane molecular dynamics. Proc. 2nd Int. Conf. Boar Semen Preserv., Beltsville, 81-93.

Bvochora, J.M., Zvauya, R. 2001. Biochemical changes occurring during the application of high gravity fermentation technology to the brewing of Zimbabwean traditional opaque beer. Process Biochem. ;37: 365–70.

Bvochora, J.M., Danner, H., Miyafuji H. & Braun, R., Zvauya R. 2004. Variation of sorghum phenolic compounds during the preparation of opaque beer. Process Biochem. 40: 1207–1213.

Cardoso, R.C.S., Silva, A.R., Uchoa, D.C. & Silva, L.D.M. 2002. Cryopreservation of canine semen using coconut extender with egg yolk and three different glycerol concentrations. Theriogenol. 59: 743–751.

Cevorsky, J., Hudecek, V., Rozkot, M. & Hercik, Z. 1999. Effects of heterospermic insemination of hybrid sows on the farrowing rate and litter size. Czech J Anim Sci. 44: 13-7.

Chen, B.X., Zhao, X.X. & Huang, Y.M. 1990. Freezing semen and AI in the Bactrian camel (*Camelus bactrianus*). In: Proceedings of the workshop “Is it Possible to Improve the Reproductive Performance of the Camel?” Paris, September 10–12, 1990, 13.

Christensen, P., Knudsen D.B., Wachman, H. & Madsen, M.T. 2004. Quality control in boar semen production by use of the FACSCount AF system. Theriogenol. 62: 1218–28.

Chung, K.T., Wong, T.Y., Wei, C.I. & Huang, Y.W., Lin, Y. 1998. Tannins and human health. A review. Critical Rev. Food Sci. Nutr. 38: 421–464.

Ciereszko, A. & Ottobre J.S., Glogowski, J. 2000. Effects of season and breed on sperm acrosin activity and semen quality of boars. Anim Reprod Sci. 64: 89-96.

Clifford, M.N. 2000. Anthocyanins – nature, occurrence and dietaryburden. J. Agri. Food Chem. 80: 1063–1072.

Colenbrander, B., Feitsma, H. & Grooten, H.J. 1993. Optimizing semen production for artificial insemination in swine. J. Reprod. Fertil. Suppl. 48: 207– 215.

Colenbrander, B., Gadella, B., Feitsma, H. & Woelders, H. 2000. Semen quality assessment, present and future. In: Johnson, L.A., Guthrie, H.D. (Eds), Boar Semen Preservation IV Proceedings. Lawrence, KS, USA: Allen Press Inc., 35-41.

Corcuera, B.D., Herna´ndez-Gil, R., De Alba Romero, C. & Mart´ın Rillo, S. 2002. Relationship of environment temperature and boar facilities with seminal quality. Liv. Stock. Prod. 74: 55-62.

Correa, M.N., Lucia, T., Alfonso, J.A.B. & Deschamps, J.C. 2002. Reproductive performance of early-weaned female swine according to their estrus profile and frequency of artificial insemination. Theriogenol. 58: 103–12.

Crabo, B.G. 1991. Preservation of boar semen. A world wide perspective. In: Johnson, L.A., Rath, D. (Eds). Boar Semen Preservation II. Proceeding of Second International and Conference on Boar Semen Preservation. Berlin: Paul Parey Scientific Publishers, 3-9.

Cross, N.L. 1996. Human seminal plasma prevents sperm from becoming acrosomally responsive to the agonist, progesterone: cholesterol is the major inhibitor. Biol. Reprod. 54: 138-145.

Curry, M.R. 2000. Cryopreservation of semen from domestic livestock. J. Reprod. Fertil. 5: 46–52.

Cyr, D.G., Finnson, K., Dufresne & J., Gregory, M. 2002. Cellular interactions and the blood-epididymal barrier. In: Robaire, B., Hinton, B.T., (Eds). The epididymis: from molecules to clinical practice. New York: Kluwer. Academic/Plenum Publishers, 103–18.

De Leeuw, F.E., Colenbrander, B. & Verkleij, A.J. 1990. The role membrane damage plays in cold shock and freezing injury. Proc. 2nd Int. Conf. Boar Semen Preserv., Beltsville, 95-104.

Delgado, C., Rosengrant, M., Steinfeld, H., Ehui & S., Courbois, C. 1999. *Livestock to 2020: the next food revolution.* Rome, Italy: UN FAO, 72.

Demuyakor, B., Ohta, Y. 1993. Characteristics of single and mixed culture fermentation of Pito beer. J. Sci .Food Agric. 62: 401–8.

Dumpala, A.M., Parker, H.M. & Mcdaniel, C.D. 2006. The Sperm Quality Index From Fresh Semen Predicts Semen Quality After Storage. Int. J. Poult Sci. 5(9): 850-855.

Esele, J.P., Fredriksen, R.A. & Miller, F.R. 1993. The association of genes controlling caryopsis traits with grain mold resistance in sorghum. Phytopathol. 83: 490–495.

Evenson, D.P., Thompson, L. & Jost, L. 1994. Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. Theriogenol. 41: 637–51.

Evenson, D.P., Larson, K.L. & Jost, L.K. 2002. Sperm chromatin structure assay: Its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J. Androl. 23: 25–43.

FAO Database. 2002: http://apps.fao.org/cgi-bin/nph-db.pl?subset=agriculture. [Accessed 12: 12: 2008]

Flowers, W.L. 1997. Management of boars for efficient semen production. *J. Reprod.* Fert. Suppl. 52: 67–78.

Flowers, W.L. 1998. Management of reproduction. In: Wiseman, J., Varley, M.A., Chadwick, J.P. (Eds), Progress in Pig Science. Nottingham: University of Nottingham Press, 383–405.

Flowers, W. L. 2002. Inseminated increasing fertilization rate of boars: Influence of number and quality of spermatozoa. *J Anim Sci* 80:E47-E53.

Foote, R.H. 2003. Fertility estimation: a review of the past experience and future prospects. Anim. Reprod. Sci. 75: 119-139.

Foxcroft, G.R., Dyck, M.K., Rui-Sanchesz, A., Novak, S., Dixon, W.T. 2008. Identifying useable semen. Theriogenol. 70: 1324-1336.

Funnell, D.L. & Pedersen, J.F. 2006. Reaction of sorghum lines genetically modified for reduced lignin content to infection by Fusarium and Alternaria spp. Plant Disease 90: 331–338.

Gadaga, T.H., Mutukumira, A.N., Narvhus, J.A. & Feresu, S.B. 1999. A review of traditional fermented foods and beverages of Zimbabwe. Int. J. Food Microbiol. 53: 1–11.

Gadea, J., Mata´s, C. & Lucas, X. 1998. Prediction of porcine semen fertility by homologous *in vitro* penetration (hIVP) assay. Anim. Reprod. Sci. 56: 95–108.

Gadea, J. & Matas, C. 2000. Sperm factors related to *in vitro* penetration of porcine oocytes. Theriogenol. 54: 1343–1357.

Gadella, B. M., Flesch, F. M., Van Golde, L. M. & Colenbrander, B.: 1999 Capacitation Induces Tyrosine Phosphorylation of Proteins in the Boar Sperm Plasma Membrane Volume 262, Issue 3, 7 September 1999, 787-792

Gagnon, C. 1995. Regulation of sperm motility at the axonemal level. Reprod. Fertil. Dev. 7: 847-855.

Garde, J.J., Del Olmo, A., Soler, A.J. & Espeso, G. 2007. Effect of egg yolk, cryoprotectant, and various sugars on semen cryopreservation in endangered Cuvier's gazelle. Anim. Reprod. Sci. 108; 384-401.

Garner, D.L., Thomas, C.A., Gravance, C. G., Marshall, C. E., De Jarnette, J.M., Allen, C.H. 2001. Seminal plasma addition attenuates the dilution effect in bovine sperm. Theriogenol. 56(1): 31-40.

Gatti, M.S.V., Ferraz, M.M.G., Ràcz, M.L. & De Castro, A.F.P. 1993. Rotavirus excretion in naturally infected pigs with and without diarrhea. Vet. Microbiol. 37(2-2): 197-190.

Gillmore, J.A., Peter, A.T. & Critser, J.K. 1998. Determination of plasma membrane characteristics of boar spermatozoa and their relevance to cryopreservation. Biol Reprod. 58: 28-36.

Graham, L.H., Bando, J., Gray, C. & Buhr, M.M. 2004. Liquid storage of Asian elephant (*Elephas maximus*) sperm at 4^{o}C. Anim. Reprod. Sci. 80: 329–340.

Gravence, C.G., Barden, D.L., Pitt, C., Vishwanath, R., Sax-Gravence, S.K. & Casey, P.J. 1999. Replicate and technician variation associated with computer aided bull sperm head morpometry analysis (ASTHMA). Int. J. Androl. 22: 77-82.

Green, C.E. & Watson, P.F. 2001. Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation. Reprod. 122: 889–98.

Green, C.E., & Watson, P.F. 2002. Adverse effect of winter overnight temperature on transported liquid boar semen. Vet. Rec. 151: 302–3.

Gry, B.B., Annette K.E., Torben, G. & Christensen, P. 2004. Increasing storage time of extended boar semen reduces sperm DNA integrity. Theriogenol. 63: 2006–2019.

Haugan, T., Reksen, O., Gröhn, Y.O., Gaustad, A.H. & Hofmo, P.O. 2004. A retrospective study on the effects of storage time in liquid boar semen on reproductive performance in Norwegian swine. Theriogenol. 64: 891-901.

Hazary, R.C., Staines, S.J. & Wishart, G.J. 2000. Assessing the efficiency of mating in broiler breeder flocks by enumerating the spermatozoa which penetrate the inner perivintelline layer over the germinal disc. Brit. Poult Sci. 41: 395-400.

Hinton, B.T. & Palladino, M.A. 1995. Epididymal epithelium: its contribution to the formation of a luminal fluid microenvironment. Microsc Res Tech 30: 67–81.

Hofmo, P.O. 2000. *In vivo* comparison of (BTS) and MR-A under Norwegian AI conditions. In: Johnson, L.A., Guthrie, H.D. (Eds). Proceeding of the Fourth International Conference on Boar Semen Preservation. Allen Press, Inc., Lawrence KS. USA, 245-6.

Holt, W.V., Abaigar, T. & Jabbour, H.N. 1996. Oestrus synchronization, semen preservation and artificial insemination in the Mohor Gazelle (Gazella dama mhorr) for the establishment of a genome resource bank programme. Reprod. 30: 67:12.

Huang, Y.T. & Johnson, R.K. 1996. Effect of selection for size of testes in boars on semen and testis traits. J. Anim. Sci. 74: 750– 760.

Huang, S.Y., Tu, C.F., Liu, S.H., Kuo, Y.H. 2004. Motility and fertility of alginate encapsulated boar spermatozoa. Anim. Reprod. Sci. 87: 111-120.

Igeur-ouada, M. & Verestegen, J.P. 2001. Long term preservation of chilled canine semen: effect of commercial and laboratory prepared extenders. Theriogenol. 55: 671–684.

Ivanova, M., Mollova, M. 1993. Zona-penetration *in vitro* test for evaluating boar sperm fertility. Theriogenol. 40: 397-410.

Johnson, L.A., Weitze, K.F., Fiser, P. 7 Maxwell, W.M.C. 2000. Storage of boar semen. Anim. Reprod. Sci. 62: 143–172.

Juonala, T., Lintukangas, S., Nurttila, T. & Andersson, M. 1998. Relationship between semen quality and fertility in 106 AI-boars. Reprod. Dom. Anim. 33: 155-158.

Katska, L., Ryňnska, B. & Smorag, Z., 1996. Effect of seminal plasma on the in vitro fertilizability of bull spermatozoa. Anim. Reprod. Sci. 44: 23–31.

Kommisrud, E., Paulenz, H., Sehested, E. & Grevle, I.S. 2002. Influence of Boar and Semen Parameters on Motility and Acrosome Integrity in Liquid Boar Semen Stored for Five Days. Acta Vet. Scand. 43: 49-55.

Kozink, D, M., Estienne, M, J., Harper, A, F. & Knight, J, W. 2002. The Effect of Lutalyse on the Training of sexually inexperienced boars for semen collection. Theriogenol. 58: 1039-1045.

Kuster, C.E. & Althouse, G.C. 1999. The fecundity of porcine semen stored for 2 to 6 days in Androhep and XCELL extenders. Theriogenol. 52(3): 365-376.

Laforest, J.P.& Allard, D. 1996. Comparison of four extenders for long-term storage of fresh boar semen. Reprod. Dom. Anim. 31: 275–6.

Lai, B.T., Chin, N.W., Stanek, A,E., Keh, W. & Lank, K,W. 1993. Quantitation of and Intracellular localization of the 85K heat shock protein by using monoclonal ang polyglonal antibodies. Mol. Cel. Biol. 4: 2802-2810.

Lambert, E., Williams, D.H., Lynch, B.P., Hanrahan, T.J., Mcgeady, T.A., Austin, F.H., Boland, M.P. & Roche, J.F. 1991. The extent and timing of prenatal loss in gilts. Theriogenol. 36: 655-665.

Lanzafame, F., Chapman, M.G., Guglielmino, A., Gearon, C.M. & Foreman, R.G. 1994. Pharmacological stimulation of sperm motility. Hum. Reprod. 9: 192-199.

Levis, D.G. 2000. Liquid boar semen production; current extender technology and where do we go from here! In: Johnson, L.A., Guthrie, D.H. (Eds). Proceeding from Fourth International Conference on Boar Semen Preservation. Lawrence, K.S., USA: Allen Press, Inc., 121-8.

Lin, C.L., Ponsuksili, S., Tholen, E., Jennen, D.G.J., Schalender, K. & Wimmers, K. 2006. Candidate gene makers for sperm quality and fertility of boar. Anim. Reprod. 92: 349-363.

Long, C.R., Dobrinsky, J.R. & Johnson, L.A. 1999. *In vitro* production of pig embryos: comparisons of culturemedia and boars. Theriogenol. 51: 1375–1390.

Louis, G.F., Lewis, A.J., Weldon, W.C., Ermer, P.M., Miller, P.S., Kittok, R.J. & Stroup, W.W. 1994. The effect of energy and protein intakes on boar libido, semen characteristics, and plasma hormone concentrations. J. Anim. Sci. 72: 2051– 2060.

Malmgren, L. 1997. Assessing the quality of raw semen: a review. Theriogenol. 48: 523-530.

Manjunath, P., Bergeron, A., Lefebvre, J. & Fan, J., 2007. Seminal plasma proteins: functions and interaction with protective agents during semen preservation. In: Roldan, E.R.S., Gomendio, M. (Eds), Spermatology, SRF Supplement 65. Nottingham University Press, Nottingham, UK, 217–228.

Maxwell, W.M. & Johnson LA. 1997. Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling, or cryopreservation. Mol. Reprod. Dev. 46: 408–18.

Maxwell, W.M.C. & Johnson LA. 1999. Physiology of spermatozoa at high dilution rates: theinfluence of seminal plasma. Theriogenol. 52: 1353–62.

Montanari, L., Perretti, G., Natella, F., Guidi, A. & Fantozzi, P. 1999. Organic and phenolic acids in beer. Lebensmittel-Wissenchaft und-Technologie 32: 535–9.

Morrow, M. 1994. The Swine AI Book. Raleigh: Morrow Publishing: 51-66.

Musa, B., Sieme, I., Merkt, H. & Hago, B.E.D., 1992. Artificial insemination in dromedary camels. In: Proceedings of the First International Camel Conference, Dubai, UAE, February 2–6. 179–182.

Nwanguma, B.C. & Eze, M.O. 1996. Changes in the concentrations of the phenolic constituents of sorghum during malting and mashing. J. Sci. Food. Agric. 70: 162–6.

Obizoba, I.C. & Atii, J.V. 1991. Effect of soaking, sprouting, fermentation and cooking on nutrient composition and some anti-nutritional factors of sorghum (*Guinesia*) seeds. Plant Foods Hum. Nutr. 41: 203–12.

Olson, G.E., NagDas, S.K. & Winfrey, V.P. 2002. Structural differentiation of spermatozoa during post-testicular maturation. In: Robaire, B., Hinton, B.T., (Eds). The epididymis: from molecules to clinical practice. New York: on a global basis. Anim. Reprod. Sci. 28: 247–257.

Parks, J.E., Lynch, D.V. 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. Cryobiol. 29: 255–66.

Paulenz, H., Soderquist, L., Perez-Pe, R., Berg, K.A., 2002. Effect of different extenders and storage temperatures on sperm viability of liquid ram semen. Theriogenol. 57: 823–836.

Pěrez, M.C., Sanchez, R., Palacio, M., Pursel, V.G., Perez Garcia, T., Martin, R.S. 1991. Effects of dilution rate on the motility and acrosome morphology of boar spermatozoa stored at 15°C. *Reprod.* Dom. Anim. 26: 112-116.

Pěrez, R., Cebria´n-Pe´rez, J.A., Muin˜o-Blanco, T. 2001. Semen plasma proteins prevent cold-shock membrane damage to ram spermatozoa. Theriogenol. 56: 425–34.

Popwell, J.M., Flowers, W. L. 2004. Variability in relationships between semen quality and estimates of *in vivo* and *in vitro* fertility in boars. Anim. Rep. Sci. 81: 97-113.

Reed, H.C.B. 1990. Commercial requirements for an effective fresh semen diluent. In: Proceedings of the Second International Conference on Boar Semen Preservation, 255–70.

Rigau, T., Piedrafita, J., Reverter, A., Canal, M. & Rodríguez-Gil, J.E. 1996. The rate of L-lactate production: a feasible parameter for the fresh diluted boar semen quality analysis. Anim. Reprod. Sci. 43: 161–72.

Robertson, L., Bailey, J.L., Buhr, M.M. 1990. Effects of cold shock and phospholipase A2 on intact boar spermatozoa and sperm head plasma membranes. Mol. Reprod. Dev. 26:143-149.

Rodriguez-Martinez, H., Tienthai, P., Suzuki, K., Funahashi, H., Ekwall, H. & Johannisson, A., 2001. Involvement of the oviduct in sperm capacitation and oocyte development in pigs. Reprod. Suppl. 58: 129–145.

Rogel-Gaillard, C., Hayes, H., Bourgeaux, N. & Chardon, P. 2001. Assignment of two new loci for gamma 1 porcine endogenous retroviruses (gamma 1 PERV) to pig chromosome bands 2q21 and 11q12 by in situ hybridization. Cytogenet. Cell Genet. 95: 112–3.

Roldàn, R. S. 1998. Signal transduction during mammalian sperm acrosomal exocytosis. In: Lauria A, Gandolfi. 1998). Phospholipase D and exocytosis of the ram sperm acrosome 1210(1): 48-54.

Rooney, L.W. 2004. Sorghum improvement: Integrating traditional and new technology to produce improved genotypes. Adv. Agron. 83: 37–109.

Rota, A. 1998. Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden. 143–149.

Rothschild, M.F. 1996. Genetics and reproduction in the pig. Anim. Reprod. Sci. 42: 143–151.

Rozeboom, K. J. 2001. *Evaluating boar semen quality* [http://www.aspajournal.it/archivio/pdf_2007/10_PIGS_production.pdf]. (ANS 00-812S). Accessed 12: 05:2008)

Ruiz-Sánches, R. Control de calidad esperma´tica. 1991. Anaporc. 104: 27–33.

Ruiz-Sánches, R. Donoghue, A.L., Novak, S., Dyk, M.K., Cosgrove, J.R., Dixon, W.T. & Foxcroft, J.R. 2005. The predictive value of routine semen evaluation and IVF technology for determining relative boar fertility. Theriogenol. 66: 736-748.

Saito, K., Kinoshita, Y. Kanno, H. & Iwasaki, A. 1996. The role of potassium ion and extracellular alkalization in reinitiation of human spermatozoa preserved in electrolyte-free solution at 4°C. Fert. Steril. 65: 1214-1216.

Sancho, S., Pinart, E., Briz, M., Garcia-Gill, N., Badia, E., Bassols, J. Kádár, E., Prenuda, A., Bassalleu, A., Yester, M., Coll, M.G. & Bonet, S. 2004. Semen quality of postpubertal boar during increasing and decreasing natural photoperiods. Theriogenol. 62: 1271-1282.

Santiago-Moreno, J., Coloma, M.A., Toledano-Díaz, A., Gómez-Brunet, A., Pulido-Pastor, A., Zamora-Soria, A., Carrizosa, J.A., Urrutia, B. & López-Sebastián, A. 2008. A comparison of the protective action of chicken and quail egg yolk in the cryopreservation of Spanish ibex epididymal spermatozoa. Cryobiol. 57: 25–29.

[SAS] Statistical Analysis System. 2002. Statistical Analytical Institute Inc., Cary, North Carolina, USA.

Sase, I., Miyata, H., Corrie, J.E.T., Craik, J.S. & Kinosota Jr., K., 1995. Real time imaging of single uorophores on moving actin with an epi uorescence microscope. Biophys. J. 69: 323-328.

Silva, J.R.V., Lucci, C.M., Carvalho, F.C.A., Báo, S.N., Costa, S.H.F., Santos, R.R. & Figueiredo, J.R. 2000. Effect of coconut water and Braun-Collins solutions at different temperatures and incubation times on the morphology of goat preantral follicles preserved in situ. Theriogenol. 54, 809–822.

Singh, V., Moreau, R.A. & Hicks, K.B. 2003. Yield and phytosterol composition of oil extracted from grain sorghum and its wet-milled fractions. Cereal Chem. 80(2): 126–129.

Smital, J., De Sousa, L. L. & Mohsen, A. 2004. Differences among breeds and manifestation of heterosis in AI boar sperm output. Anim. Reprod. Sci. 80: 121–130.

Snedecor, G.W. & Cochran, W.G. 1980. *Statistical Methods.* 7th ed. The Iowa State USA: University Press.

Surai, P.F., Speake, B.K., Noble, R.C. & Mezes, M. 1999. Species-specific differences in the fatty acid profiles of the lipids of the yolk and of the liver of the chick. J. Sci. Food. Agric. 79: 733–736.

Suriyasomboon, A., Lundeheim, N., Kunavongkrit, A. & Einarsson, S. 2004. Effect of temperature and humidity on sperm production in Duroc boars under different housing systems in Thailand.

Tardif, S., Laforest, J.P., Corrnier, N. & Bailey, J.L. 1999. The importance of porcine sperm parameters on fertility *in vivo*. Theriogenol. (52): 447-459.

Taylor, J.R.N., Dewar, J. Taylor, J. & Ascherraden, V. 1997. Factors affecting porridge making quality of South African sorghums. J. Sci. Food Agric. 73: 464-470.

Taylor, J.R.N. 1998. Sorghum malting science and malt assessment. Presented at the Workshop on Sorghum End Use Quality Assessment, University of Pretoria, Department of Food Science, Pretoria, South Africa, 1-4.

Trimeche, A., Anton, M., Renard, P., Gandemer, G. & Tainturier, D. 1997. Quail egg yolk: a novel cryoprotectant for the freeze preservation of Poitou jackass sperm. Cryobiol. 34: 385–393.

Umesiobi, D.O., Iloeje, M.U., Berepubo, N.A. & Anyanwu, G.A. 1999. Influence of sperm dose and time of insemination on the conception rate of sows. Delta Agric. 6: 206 - 212.

Umesiobi, D.O. & Iloeje, M.U. 1999. Effect of sexual teasing and diurnal period of semen collection on reaction time and semen characteristics of Large White boars. J. Sustain. Agric. Environ. 1(2): 231-235.

Umesiobi, D.O. 2000a. Effect of egg yolk, coconut water and fresh raphia palmwine extenders on the viability of boar sperm during storage at 50°C. J. Agric. Rural Dev. 1(1): 98 - 106.

Umesiobi, D.O. 2000b. Animal Production I. Basic Principles and Practices. Bean Blaise Publication, Owerri: Nigeria, 129.

Umesiobi, D. O., Iloeje, M.U., Anyanwu, G.A., Herbert, U. & Okeudo, N.J. 2000. Relationship between sow fecundity and sexual repertoire of the boar. J. Agric. Rural Dev. 1(1): 114 - 119. Umesiobi, D. O. 2001. Effects of season and diurnal period on the body physiology of Nigerian sheep breeds. Trop. J. Anim. Sci. 4(1): 59 - 67.

Umesiobi, D.O., Iloeje, M.U. & Berepubo, N.A. 2002. Artificial insemination in sows using Guelph and two local semen extenders. Nig. J. Anim. Prod. 29(1): 121 - 126.

Umesiobi, D.O. 2004. Functional integrity of boar spermatozoa and sow fertility using raphia (*Raphia hookeri*) palmwine plus 'Nche' (*Saccoglotis gabonensis*) urban extender. J. Appl. Anim. Res. 26: 13-16.

Umesiobi, D.O., Kalu, U., Ogundu, U., Iloeje, M.U., Anyanwu, D.C. & McDowell, L.R. 2004. Fertility studies on two methods of libido maintenance in West African Dwarf rams. J. Anim. Vet. Advances 3(2): 81-84.

Umesiobi, D.O. 2006a. Effect of oral administration of Clomiphene citrate on sperm viability and fertility of boar semen. J. Appl. Anim. Res. 30: 167-170.

Umesiobi, D.O. 2006b. The effect of hemi-orchidectomy on reproductive traits of boars. South Afr. J. Anim. Sci. 36(3): 181-188.

Umesiobi, D.O. 2007. Measures of libido and their relation to testicular hypertrophy and fertilizing competence in boars. J. Anim. Sci. 85(Suppl. 1): 815.

Umesiobi, D.O. 2008a. Measures of servicing capacity of boars and their effects on subsequent fertility in artificially inseminated gilts. J. Appl. Anim. Res. 34: 9-12.

Umesiobi, D.O. 2008b. Effects of sexual stimulation of boars on the fertility and fecundity rates in sows. The Philipp. Agric. Scientist 91(1): 379-385.

Umesiobi, D.O. 2008c. Supplemental Vitamin E: A requirement for optimizing fecundity rates and litter size in sows. The Philipp. Agric. Scientist 91(2): 187-194.

Umesiobi, D.O. 2009. Vitamin E supplementation and effects on fertility rate and subsequent body development of their weanling piglets. J. Agric. Rural Dev. Trop. Subtrop. 110(2): 155-168.

Van Groenland, G.L. 1993. Alojamiento de verracos para IA. Anaporc 128: 57–59.

Vishwanath, R., Shannon, P. 2000a.: Do sperm cells age? A review of the physiological changes in sperm during storage at ambient temperature. Reprod. Fertil. 30: 167-170.

Vishwanath, R., Shannon, P. 2000b. Storage of bovine semen in liquid and frozen state. Anim. Reprod. Sci. 62: 23–53.

Waberski D. 1990. Spermienmotilität und Morphologie in ihrer Auswirkung auf die Fruchtbarkeit von Besamungsebern in Feldversuchen. Tieräztl. Prax., 18: 591–594.

Waberski, D., Dirksen, G., Weitze, K.F., Leiding, C. & Willmen, T. 1990. Effect of semen quality on fertility results of stored liquid boar semen – a field trial. In: Proceedings of the Second International Conference on Boar Semen Preservation, 375–8.

Waberski, D., Meding, S., Dirksen, G., Weitze, K.F., Leiding, C. & Hahn, R. 1994a. Fertility of long-term stored boar semen: Influence of extender

(Androhep and Kiev), storage time and plasma droplets in the semen. Anim. Reprod. Sci. 36: 145-151.

Waberski, D., Weitze, K.F., Leitmann, C., Lubbert zur Lage, W., Bortolozzo, F.P., Willmen, T. 1994b. The initial fertilizing capacity of long-term stored liquid boar semen following pre and postovulatory insemination. Theriogenol. 41: 1367–77.

Waberski, D., Weitze, K.F., Lübbert zur Lage, W., Bortolozzo, F.P., Willmen, T., Petzoldt, R. 1994c. The initial fertilizing capacity of longterm stored liquid boar semen following pre-and postvoluntary insemination. Theriogenol. 41: 1367-1377.

Weitze, K.F. 1990. Long term storage of boar semen. Reprod. Dom. Anim. Supp. 1: 231-53.

Wildt, D.E. 1992. Genetic resource banks for conserving wildlife species: justification, examples and becoming organized.

Willenburg, K.L., Miller, G.M. & Rodriguez-Zas, S.L. 2003. Effect of boar exposure at time of insemination on factors influencing fertility in gilts. J. Anim. Sci. 81: 1-8.

Xu, X., Seth, P.C., Harbison, D.S., Cheung, A.P. & Foxcroft, G.R. 1996. Semen dilution for assessment of boar ejaculate quality in pig IVM and IVF systems. Theriogenol. 46: 1325–1337.

Xu, X., Pommier, S., Arbov, T., Hutchings, B., Sotto, W. & Foxcroft, G.R., 1998. *In vitro* maturation and fertilization techniques for assessment of semen quality and boar fertility. J. Anim. Sci. 76: 3079–3089.

Yanangimachi, R. 1994. Mammalian Fertilization. In: Knobil, E., Neill, J. (Eds). The Physiology of Reproduction, 2nd ed. New York: Raven, .189-317.

Zahraddeen, R., Butswat, I.S.R., Kalla, D.J.U., Sir, S.M. & Bukar, M.T. 2005. Effect of Frequency of Ejaculation on Semen Characteristics in Two Breeds of Turkeys (Meleagris gallopavo) Raised in Tropical Environment. Int. J. Poult. Sci. 4: 217-221.

Zou, C.X. & Yang, Z.M. 2000. Evaluation on sperm quality of freshly ejaculated boar semen during *in vitro* storage under different temperatures. Theriogenol. 53: 1477–1488.