An evaluation of effect of two extenders and storage temperature on quality of Alpine goat semen processed with and without seminal plasma

'A Thesis submitted in partial fulfilment of the requirements for Masters Degree in Theriogenology of University of Nairobi'

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Declaration

"This thesis is my original work and has not been presented for a degree in any other University"

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Dedication

I dedicate this work to my wife Joyce Juma and our sons Elvis Juma and Evans Juma.

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I thank the Almighty God for being with me throughout this walk, which I must admit, was rigorous. Special thanks go to EAAPP for offering me the opportunity to study for my Masters degree. I further appreciate the Principal Secretary through the Director, State Department of Livestock for relieving me of my duties to allow me undertake my studies.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACOC: Semen sample with intact seminal plasma extended in coconut water based extender

AHITI: Animal Health and Industry Training Institute

AI: Artificial Insemination

ANOVA: Analysis of Variance

AOPT: Semen sample with intact seminal plasma extended in OPTIXcell™.

ATP: Adenosine triphosphate

AV: Artificial Vagina

BCOC: Semen sample without seminal plasma extended in coconut water based extender

BOPT: Semen sample without intact seminal plasma extended in OPTIXcell™

CASA: Computer Assisted Semen Analysis

CEO: Chief Executive Officer

CH: Chilled Semen

COC: Coconut water based extender

DF: Deep-frozen semen

df: Degree of freedom

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

EAAPP: Eastern Africa Agricultural Productivity Project

EYCE: Egg York Coagulating Enzyme

GLM: General Linear Model

K.A.G.R.C: Kenya Animal Genetic Resource Centre

kDa: kilodalton

LDL: Low-Density Lipoprotein

N: Sample size

OPT: OPTIXcell™

PLPR2:Pancreatic lipase-related protein 2

ROS: Reactive Oxygen Species

rpm: revolutions per minutes

RT: Room Temperature semen

SD: Standard Deviation

SEM: Standard Error of Mean

Sig.: Significance (statistical)

SP: Seminal plasma

Temp: Temperature

UoN: University of Nairobi

WAD: West African Dwarf buck

ABSTRACT

This study was designed to evaluate the quality of semen of Alpine goats extended in Optixcell^{TM} ((OPT), a commercially available semen extender) and coconut water based egg-yolk-containing extender (COC) with and without seminal plasma and stored under three different temperature conditions. COC was a newly formulated extender whose capability to maintain viability of goat semen was test against the commercially available OPT. Semen samples were stored either at room temperature as RT semen, chilled (CH semen), or deep-frozen in liquid nitrogen (DF semen). Viability of semen was determined using two parameters; percentage individual progressive motility of spermatozoa and percentage live spermatozoa.

Ejaculates were collected from bucks using artificial vagina. Each ejaculate from each buck was split into two portions. One portion was left intact whereas the other portion was centrifuged to remove seminal plasma. The pellet obtained after centrifugation was reconstituted to the initial volume (before centrifugation) using normal saline. Each of the two parts was further divided into two equal portions for extension with each extender. Extended semen samples were stored at room temperature (21-23^{0}C), chilled (1-4^{0}C) and deep-frozen in liquid nitrogen (-196^{0}C). Semen viability of the processed samples stored at room temperature and chilled was evaluated daily until zero values were recorded. Deep-frozen samples were examined for spermatozoa viability only once after 120 days of storage in liquid nitrogen.

The results showed that addition of either extender to goat semen was not detrimental to the spermatozoa in semen samples with or without seminal plasma. However, motility was significantly reduced within two hours following extension, in both samples with and without

seminal plasma ($p<0.05$). Each extender did not have any significant effect on percentage live spermatozoa with seminal plasma ($p>0.05$), but a significant drop in percentage live spermatozoa was observed in samples where seminal plasma was removed ($p<0.05$). There was no significant difference in the longevity of spermatozoa between the two extenders with and without seminal plasma when semen samples were stored at room temperature and chilled ($p>0.05$). The results further showed that removal of seminal plasma prolonged the shelf life of spermatozoa in each extender ($p<0.05$).

At 50% motility cut-off, semen extended in OPT and stored at room temperature maintained viability for only 3 days with seminal plasma. However, when seminal plasma was removed it performed better (5 days) similar to COC with and without seminal plasma. Semen samples stored chilled with or without seminal plasma maintained motility above 50% for at least six days in both extenders. However, three additional days were recorded when semen was extended in OPT with seminal plasma removed.

Deep-frozen semen samples extended in OPT recorded mean post-thaw viability values above 50% at 120 days while those extended in COC were all dead.

From the findings of this study, both extenders could be used in processing goat semen intended for room temperature and chilled semen use with or without seminal plasma. However, only OPT and not COC would be used to extend semen for deep freezing. Semen extended in COC maintained usable viability for the same duration (5 days) when stored at room temperature with and without seminal plasma. For chilled semen, the difference in duration with and without seminal plasma was also minimal (6 and 7 days). Therefore, it would not be necessary to remove seminal plasma from goat semen to be extended in COC, for artificial insemination, if such semen was to be used as room temperature or chilled at 1-

4^0C. However, removal of seminal plasma favoured greatly the longevity of usable viability of spermatozoa for both room temperature and chilled semen when extended in OPT.

CHAPTER 1.0: INTRODUCTION

1.1 General Background

Semen extension using extenders is a critical step in semen processing for preservation. Extenders are used to increase the volume of semen from one ejaculate, which can then be packaged in several breeding doses for artificial insemination (AI). The extended semen can be stored as room-temperature semen (RT), chilled semen (CH) or deep-frozen semen (DF). Extenders supply the sperm cells with sources of energy, control of infectious agents, protect the cells from temperature-related damage, and maintain a suitable environment for the spermatozoa to survive (Leboeuf *et al*., 2000).

Preparing a universal extender suitable for preserving semen of all animal species faces a challenge because of differences in size and shape of spermatozoa, biochemical composition of spermatozoa plasmalema, in addition to differences in liquid composition (seminal plasma) of semen of the various animal species. Even those extenders that are in use in individual animal species do not give 100% post-storage semen qualities (Purdy, 2006).

In the livestock industry, most studies to establish the ideal extender for preservation of semen dwelt on bull semen (Baracaldo *et al*., 2007; Rehman *et al.,* 2013). The same extenders have been adopted for use in other species including goats but results have been variable (Purdy, 2006). They include egg yolk-, skimmed milk- and coconut milk-based extenders (Sule *et al.,* 2007; Luzardo *et al*., 2010). In the goat, there exists interactivity between seminal plasma and milk or egg yolk that is deleterious to the sperm, a situation not observed with bovine seminal plasma (Purdy, 2006). Yet milk and egg yolk are common additives in most semen extenders. The effects resulting from interaction of goat seminal plasma and egg yolk were first demonstrated by Roy in late 1950s. He found that

spermatozoa maintained motility in egg yolk when seminal plasma was removed unlike when whole ejaculate was used. In addition, he noted that egg yolk coagulated and it was determined that an enzyme of bulbourethral gland origin was responsible for the coagulation, thus called Egg Yolk Coagulating Enzyme (EYCE). A similar interactivity was observed between milk and goat semen by Nunes and colleagues in the early 80s and the enzyme responsible, also from the bulbourethral gland, was a 50-60 kDa glycoprotein lipase called SBUIII (Pellicer-Rubio *et al.,* 1997). These effects elicited several researches on goat semen to verify the extents of the effect of the interaction.

In the 1980s, many researchers agreed with the interactive effects of seminal plasma with both milk and egg yolk. Later, some researchers found no difference in quality of semen extended in presence and absence of seminal plasma (Peterson *et al*., 2007; Jiménez-Rabadán *et al*., 2012). As a corrective measure to the largely known effect, researchers have resorted to alternative extenders that do not have either milk or egg yolk. Coconut water (Sule *et al.,* 2007) and soybean (Vidal *et al*., 2013) have been some of such alternatives. Though high concentration of coconut water has been associated with preventing free motility of spermatozoa, it has given good viability values especially for semen stored at room temperature (Sule *et al.,* 2007). Detrimental effects over frozen-thawed sperm viability however, have been recorded with ram semen when coconut water was used (Santillana and Garcia 2000; Gutierrez *et al.,* 2006).

This study compared the qualities of buck semen extended with a non-egg-yolk based commercial extender OPTIXcell™ (OPT) versus a laboratory constituted coconut water based extender (COC) which contained egg yolk. It also compared such qualities with and without removal of seminal plasma in each extender. The semen was stored under different storage temperatures namely:- room temperature semen (RT) , chilled semen (CH) and deep-

frozen semen (DF) in bid to identify the most appropriate extender to use under a given storage temperature, for preservation of goat semen.

1.2 Justification

Dairy farming in Kenya is concentrated in the high agricultural potential areas and has traditionally been based on cattle. Increase in human population and consequent land demarcation has continuously put pressure on the land resource base. Farmers have adopted dairy goat rearing due to among other reasons, ease of feeding using a variety of crop residues, fecundity, lower initial capital investment, popularity of milk due to its high nutritive value, digestibility and the fact that goat milk is considered medicinal and the resultant higher goat milk price (Sere *et al*., 2008). For these reasons, popularity of dairy goats has driven up their demand. Shortage of breeding stock, inbreeding, low uptake of costly breeding technologies among others put AI being adopted as the easily available and low cost breeding technology, for availing the much-needed animals. The cost of AI would further be reduced if extenders were made from locally available materials like coconut water instead of importing them, which is the current trend in Kenya.

This study was designed to evaluate viability of goat semen extended in two extenders; including the newly formulated coconut water based extender, on stored buck semen in presence and absence of seminal plasma, under different storage temperatures. The target was to identify the best extender for buck semen stored with or without the plasma for use at room, chilled or frozen temperatures as a step towards enhanced dairy goat productivity.

1.3 General objective

To evaluate effect of extenders and seminal plasma on quality of stored buck semen for enhanced dairy goat productivity

1.4 Specific objectives

1. To compare the quality of stored buck semen processed with two extenders (COC and OPT) with and without seminal plasma
2. To determine the effect of temperature of storage on viability of goat semen extended with two extenders (COC and OPT)

CHAPTER 2.0: LITERATURE REVIEW

The history of AI dates back to 1300s when it was reportedly used in breeding Arabian horses (Foote, 2002), but AI became more viable between 1940 and 1950 with the discovery of cryopreservatives like egg yolk by Phillips and Lardy in 1940 and glycerol by Polge in 1949 (Foote, 2002). Milk as an extender was first reported by Michajilov in1950 (Foote, 2002). The use of AI for breeding took root in cattle. Since then, semen collection, extension, and storage for use in AI have widely been studied in farm animals including goats.

2.1 Semen collection in the buck

Like in other farm animals, standard ways of semen collection and evaluation including massage of the penis, manual stimulation of accessory sex glands, intra-vaginal condom, intra-vaginal collection post coitus, electro-ejaculation, or use of artificial vagina (AV) is practised in the goat (Austin *et al.*, 1986). Ejaculates collected by AV in the goat have been shown to have a greater semen volume and quality after thawing, as compared to other methods (Jiménez-Rabadán*et al.*, 2012). Volume and quality of semen though, is also influenced by among other factors sexual maturity and behaviour, social environment and management conditions and method and frequency of semen collection from the buck (Leboeuf *et al.*, 2000). This indicates that bucks of similar sexual maturity as dictated by breed and age, reared under similar management and social environment, would be expected to produce ejaculates whose volumes and quality fall within a similar range.

Collecting semen from bucks through the AV method of semen collection involves introduction of the penis into the open lubricated end of an artificial vagina while the male attempts to mount a teaser animal. The method has an advantage of the fact that AV simulates a similar environment experienced by the buck during natural mating. Lubricated vaginal folds made by the rubber liner of the AV accompanied with warmth from warm water

placed between the outer and inner jackets helps in achieving this probably making it the method of choice. Semen is usually collected in a graduated glass tube cushioned from light and environmental temperature variations.

2.2 Semen evaluation parameters in the buck

Following collection, several semen parameters are evaluated including volume, colour, sperm motility, concentration of spermatozoa, integrity of the plasma membrane and acrosomal membrane, mitochondrial activity and DNA fragmentation index in the goat. Semen volume has been found to range between 0.5-2mL, colour from whey-white to creamy yellow, motility from 70-90% while concentration from 2-5x10^9/mL (Mushtaq *et al.*,2007; Jiménez-Rabadán *et al*., 2012). It is common practice to read and record semen volume and colour immediately following collection (Gundogan *et al*., 2011; Udeh and Oghenesode, 2011).

In semen collection centres, evaluation of these parameters is important since they are a factor of fertility of semen, which is further important in the success of artificial breeding. For purposes of post storage comparison of viability parameters, such parameters are analyzed and recorded prior to extension usually, within 10 minutes of collection and at 37ºC (Gundogan *et al*., 2011). After recording ejaculate volume and colour, it is relatively easier to analyze integrity of membrane and sperm motility using light microscope or phase contrast microscopy as indicators of viability in a simple laboratory (Gundogan *et al.*, 2011).

2.2.1 Ejaculate volume measurement

Volumes of ejaculates vary greatly among different animal species and within the same species. Such intra-species variation has been associated with among other factors, breed, environmental temperature, age, level of nutrition, disease, libido, and method of semen collection (Mushtaq *et al.*, 2007). In the goat, an average of 0.5-2.0 mL per ejaculate has

been considered adequate for semen collected for purposes of processing in the laboratory (Mushtaq *et al.,* 2007). Though use of specialized semen collection tubes that improve on accuracy in small animals like the ram and buck have been recommended by Lorton (2014), graduated centrifuge tubes are still widely used in Kenya.

2.2.2 Evaluation of ejaculate colour and spermatozoa concentration

The colour of goat semen ranges from whey-white to cream. The concentration of spermatozoa ranges from 2-5x10^9cells/ millilitre of ejaculate (Mushtaq *et al.,* 2007). Semen colour has been associated with concentration in the goat. Whey-white semen has been postulated to have less than 0.1x10^9 sperm cell/mL, milky having 0.5-1.0x10^9cells/mL while creamy having 2.5-≥4x10^9cells/ mL (Mushtaq*et al.,* 2007). Visual inspection of colour of ejaculates has been used to estimate the number of doses of extended semen in situations where semen availability is not limited, yet this method has been considered the most inaccurate (Lorton, 2014). Measuring the concentration of spermatozoa in an ejaculate is on the other hand a fundamental and important step in semen evaluation and processing for subsequent purpose of AI since accuracy is improved. More accurate methods for estimation of sperm concentrations like, hemocytometry, spectrophotometry (e.g. the Karras Spermiodensimeter), microcells, plate reader, image analysis, and flow cytometry have been elaborately reviewed (Kumar *et al*.,2013; Lorton, 2014). Of these, hemocytometric-counting chamber has been the 'gold standard'. Variation in data using this method has been associated with manufacturers, type of the chamber among other factors including technician experience, number of extensions, and number of chambers counted (Kumar *et al*., 2013). As an error correction measure a minimum of four counts are made for each extended sample in conjunction with a standardized method of counting. Nonetheless, evaluation of samples using this method is time-consuming – approximately 15-20 min/chamber making the method

not practical where there are many samples to be evaluated in a single day (Lorton, 2014). A newer method that utilizes flow cytometry technique has been suggested by some researchers to be the 'new gold standard' for its better accuracy and ability to be used for evaluating semen of several animal species including stallion (Morrel *et al*., 2010), bulls (DeJarnette and Lefevre, 2008) and trout (Nynca and Ciereszko, 2009). However, this method also consumes time. Spectrophotometric methods are routinely used in many of the AI laboratories throughout the world in estimating sperm counts and have the advantage of completing the estimation in a short time (30-60s) (Lorton, 2014). Spectrophotometry utilizes a procedure of light penetration through semen for estimation of concentration of spermatozoa (Kumar *et al*., 2013). Photometers/ spectrophotometers are calibrated to give semen concentration values against optical concentration of the semen. Concentrations of samples are read in light absorbance units (0.0-2.0) or percentage light transmittance (0-100%). Either of the method has been shown to have low accuracy on readings on the lower (<0.2 absorbance and <10% transmittance) and upper (>1.8 absorbance and >90% transmittance) ends due to combination of too few or too many cells plus other factors that may alter passage of light through a semen sample like amount of seminal plasma (Lorton, 2014). To correct this low accuracy problems, many spectrophotometers have predetermined curves that calculate sperm/ml while others provide a reading that is used to convert the reading into sperm/ml. The accuracy of the machine is based on a standard curve generated from multiple semen samples from the same species (Lorton, 2014). Extended semen is used with some photometers while others do not require semen extension. This concept and technique of photometry has been improved over years since 1939 (Lorton, 2014). One of the modern photometric semen concentration measuring devices that offer rapid estimation of number of spermatozoa in an ejaculate is

Accucell^{TM} - IMV Technologies. This device was found to be reliable, accurate and precise thus recommended for routine photometry measures of raw semen (Camus *et al*, 2011).

2.2.3 Estimation of motility of spermatozoa

The first sperm motility was observed by Leeuwenhoek and Hartsoeker in the 17th century through microscopy. There have been modifications and improvements in the types of microscopes used for visual observation of spermatozoa since then, from compound microscopes through dark-field microscopes, bright-field microscopes and in the most recent years, computer assisted semen analyzer (CASA) that analyzes digital images. Beyond mid 20th century Rothschild and Elliott had described a 2s timed-exposure photographic procedure that utilized dark field microscopy to measure percentage of progressively motile spermatozoa. Further improvements resulted to bright-field microscopy (Parish and Foote, 1987). Video recordings from these microscopes were replayed onto standard television monitors and simultaneously observed by three to four investigators who independently and blindly evaluated sample motility. These procedures enabled workers to count tracks of motile spermatozoa in addition to counting non-motile ones from the television screens (Lorton, 2014). The technique though widely used into late 20th century in many Andrology laboratories; its advanced modifications could not eliminate its subjectivity. More recent ways of assessing sperm motility utilize real-time kinemetric analysis using the CASA system. The system has advantage of increased data objectivity, on total and progressively motile spermatozoa compared to visual microscopic analyses, but its use is limited due to high initial and maintenance cost and as such the visual microscopic system still remain the most popular means of motility analysis (Lorton, 2014).

Using the visual microscopic system the vigor of mass spermatozoa motion can be assessed. Spermatozoa in an ejaculate sample move in recurrent swirling motion as a mass that form

waves as observed under low objective magnification. In the goat, mass activity can then be categorized on numerical scale of 0-5 being 0: no movement and 5: strong wave movement (Burcu *et al.,* 2009; Jiménez-Rabadán *et al*., 2012). Other workers have graded the motion as very good (++++, vigorous swirls), good (+++, slow swirls), fair (++, no swirls but generalized oscillation) or poor (+, sporadic swirls) (Mushtaq *et al,* .2007).

Individual progressive motility of goat spermatozoa is widely used to test semen quality since spermatozoa have to individually be able to move to the fertilization site in the female for a successful AI. Percentage progressive forward motility from extended semen samples are estimated by rapid observation of 8-10 low power (x400) microscope fields consistently by one person to eliminate individual variation (Burcu *et al.,*2009).The mean of observations is then recorded as the motility score (Soe Win Naing *et al.*, 2010). It entails observation of spermatozoa as they move across a microscopic field. The observer notes how many spermatozoa progressively move across the field out of every ten spermatozoa observed. Several fields are observed to improve on accuracy.

2.2.4 Evaluation of plasmalema integrity

Semen from goats like in other farm animals contains some abnormal spermatozoa yet this does not affect fertility unless the percentage abnormalities are above 20% (Mushtaq *et al.,* 2007). Plasmalema integrity of sperms is one of the most important viability parameters commonly evaluated. Several methods of plasmalema integrity have been employed in different animal species. One of the methods is use of stains. This has been used in bulls (Brito *et al.,* 2003), boars (Fraser *et al*., 2001) and stallions (Brinsko *et al*., 2011).The second method that has been used involves incubation of spermatozoa in hyper-osmotic media and has been used in rams (Curry and Watson, 1994), and in stallions (de la Cueva *et al*., 1997). The third method involves incubation of semen in hypo-osmotic media and this has been used

in bulls (Quintero-Moreno *et al*., 2008), dogs (Pinto and Kozink, 2008), donkeys (Rota *et al*., 2010), in rams (Curry and Watson, 1994), stallions (Almin *et al*., 2010) and in turkeys (Donoghue*et al.*, 1996). Of these methods staining has been explored more. Various dyes react differently with sperm cells in general. For instance, all cells stain with rose Bengal or Bengal red and are therefore useful for morphological studies (Hackett and Macpherson, 2014). Water soluble halogenated derivatives of fluorescein for instance eosin B, eosin Y and erythrosine B were found to stain dead spermatozoa in late 1940s by Mayer and colleagues (Hackett and Macpherson, 2014). A decade later Bishop and Smiles and Van De mark and colleagues used fluorescent dyes for staining spermatozoa in opaque media (Hackett and Macpherson 2014). As further reviewed by Hackett and Macpherson more recently, stains have been used in flow cytometry for instance the SYBR-14 and PI stain combination that is commercially available in a kit for use with fluorescent microscope. NucleoCounter® SP100™ that uses fluorescent stains is in recent times preferred for use in Andrology labs due to its simplicity and assay speed (Lorton, 2014). Methods that include the use of flow cytometry are however expensive and require specialized training, leaving staining methods that use bright-field microscopy more popular for use in determining plasmalema integrity (Lorton, 2014).

Differential stains have widely been used in Andrology laboratories since Devereu and Tanner used the technology in late 1920s.The method involves use of two stains to differentiate live from dead spermatozoa. Since then, Eosin B-opal blue stains have been used successfully in sheep and was suggested for use in other mammals but later failed with human sperm (Hackett and Macpherson, 2014). Eosin B-Aniline Blue has been used with acceptable success yet some cells were found not to stain at a pH of 7.2 and gave poor results with freeze-thawed semen extended in milk (Hackett and Macpherson, 2014). Most

commonly used differential stains include eosin-nigrosin. Nigrosin provides dark background while eosin stains the cytoplasm of the spermatozoa pink if the plasmalema is broken. Such sperm cells whose cytoplasm stain pink are considered dead (Kulaksiz *et al.*, 2013). The technique has been found useful over several years. The technique was first used by Blom and Williams and Pollack in 1950 but 5% eosin was found to be toxic to spermatozoa while 10% was not (Hackett and Macpherson, 2014). It was later confirmed that eosin tends to diffuse into previously unstained cell (Emmens and Blackshaw, 1956). As a result of further research Blom's work was modified and Eosin Y was used instead. Eosin Y used at 1% with 5% Nigrosin was found to be a good combination that worked well at a wide range of pH (6.4-8.5) and semen extension rates (1:1-20:1) as differential stains (Hackett and Macpherson, 2014).

The ratio of live to dead spermatozoa is not only important in pre-extended semen to help in making decision on whether such semen should be processed, but also in assessing the quality of stored semen awaiting use for AI. It is important to note that though sperm plasmalema integrity assays are viability tests, at the time of the assay, loss of some spermatozoa plasmalema integrity may only be indicative that they will not survive *invitro* or *invivo* as long as other cells (Lorton, 2014).

2.2.5 Determination of final volume of the extender

Semen extension is an important step in goat semen processing .The efficiency of AI (fertility rate and prolificacy) is directly dependent on the quality of semen doses and on the number of spermatozoa used for insemination (Camus *et al*., 2011). The number of the spermatozoa on the other hand, is dependent on how much extender is added to neat semen. The question that arises is just how much extender shall we add to an ejaculate during processing and what is the rationale of reaching the particular volume of extender? The baseline fact is that the

number of spermatozoa deposited in the reproductive tract of the doe should be sufficient to bring forth a conception. A lot of research work has been done in this area and 150 million sperm cells per dose of semen have been considered adequate to achieve acceptable conceptions (The French Paillette Technique, www.imv-technologies.com)

Two methods exist for determining the extension rate: gross extension and extension by determination of semen concentration. In gross semen extension, parameters like volume, opacity, turbidity, and colour are used to determine how much extender to add to an ejaculate and no microscope or photometer is used to determine sperm concentration in this system (Ritar *et al*., 1990a). Extension rates are based on previous knowledge of normal ranges for total sperm output in an ejaculate. For instance, if it is known that goats produce an average of 2-5 billion sperm cells in a 2mL ejaculate, and about 140-150 million sperm cells are required per dose, then the number of doses required can be calculated (7-17 doses) and used to estimate the volume of extender to be added in relation to volume, colour, turbidity, and opacity of the ejaculate. Extension rates of 1:1–1:23 (v/v; semen to extender) have been used (Evans and Maxwell, 1987; Ritar *et al*., 1990b). This blind extension however obviously has low accuracy and repeatability.

Determining concentration of spermatozoa in an ejaculate is a more precise and repeatable way of determining the volume of extender. If semen concentration, the effective dose, and ejaculate volume are known, then the volume of extender can be determined through a mathematical calculation. $Extender\ volume = (ejaculate\ volume \times concentration \div number\ of\ cell\ at\ insemination) - ejaculate\ volume$ (Menchaca *et al*., 2005). Each time such calculation is done it is kept in mind that there will be loses during processing and storage of semen. Compared to fresh semen, cooled semen suffer decreased motility and

morphological integrity, as well as a decline in survival in the female reproductive tract, reduction in fertilizing ability and increased embryonic loss (Menchaca *et al*., 2005; Blash *et al*., 2000). Such damages are less pronounced in ram semen extended and chilled than in frozen-thawed (Aisen *et al.*, 2002). For instance, it is widely known that spermatozoa begin suffering cold shock from a storage temperature of below 17^0C and that by the beginning of deep freezing, 50% of the spermatozoa die of the same cold shock. During calculations therefore, the concentration in a single dose is commonly doubled to take care of this, for semen to be stored deep-frozen. In a busy laboratory, such calculations can be time consuming and to deal with this there are conversion tables that help speed up the process. Further, some photometric machines like Accucell^{TM} have automated ways of providing information on the volume of extender to be added and even expected number of doses from an ejaculate.

2.3 Semen extension

Gadea (2003) described the term extender to mean the aqueous solution for increasing the volume of the ejaculate to the required dose and while preserving the functionality of the sperm cells to maintain fertility. Apart from increasing the volume, extenders are not only a source of energy to sperm cells, but also provide protection against temperature-related damage in addition to maintaining a suitable environment for temporary survival of the spermatozoa (Purdy, 2006; Gadea, 2003). Components of extenders have been investigated separately, and in combination with focus on maximizing sperm longevity, viability and fertilizing capability (Purdy, 2006). In the goat, detrimental effects that results from toxic enzymatic reaction of seminal plasma and common components of extenders like egg yolk and milk has elicited research in bid to develop appropriate extenders.

2.4 Goat semen extenders

Semen extenders for goats like for other farm animals, include among other components, non-penetrating cryoprotectants like milk or egg yolk, penetrating cryoprotectants like glycerol, ethylene glycol, or dimethyl sulfoxide (DMSO), used individually or in combination (Kundu *et al*., 2000; Leboeuf *et al*., 2000). Cryoprotectants protect sperm cells from cold shock that result from cooling, freezing, and thawing (Purdy, 2006). Those classified as penetrating cryoprotectants are basically solutes which reduce water crystallization within the cells by causing dehydration thus reducing intracellular ice formation that would injure the sperms (Purdy, 2006). After sometime following semen extension, the cryoprotectants and water equilibrate to achieve intra- and extra-cellular isotonicity (Amann, 1999). The penetrating cryoprotectants also cause increased plasmalema fluidity through rearrangement of membrane lipids and proteins, which results in an increased ability of spermatozoa to survive cryopreservation (Holt, 2000). They in addition dissolve sugars and salts in the extenders.

Non-penetrating cryoprotectants protect the sperm plasma membrane from outside by modifying the plasma membrane and lowering the freezing temperature of the extender (Amann, 1999).The most common non-penetrating cryoprotectants that have been used in preservation of goat semen include2–20% egg yolk (Tuli and Holtz, 1994) and non-fat skim milk (10%, w/v) (Leboeuf *et al.*, 1998).These two components have been used for years as the basic component of extenders for preservation of semen at different storage temperatures taking advantage of their nutritive values. Lots of research work has continued with goat semen preservation given the known fact that both egg yolk and milk have detrimental effect to the survival of spermatozoa during storage.

Other non-penetrating cryoprotectants include sugars like glucose, lactose, raffinose, saccharose and trehalose (Corteel, 1974; Evans and Maxwell, 1987). Of the sugars fructose, glucose and lactose are readily utilized for respiration and provision of osmotic balance in addition to cryoprotection, with fructose having the greatest molar concentration in neat goat semen (Aboagla and Terada, 2003), making it the primary substrate for glycolysis (Pellicer-Rubio *et al*., 1997).

Buffers that have commonly been incorporated in extenders include Tris or Test. Large changes in pH result in sperm damage thus controlling fluctuation in pH of semen helps in sustaining the viability and fertilizing potential of sperm (Liu *et al.,* 2016). In nature, seminal plasma plays this role through its components thus necessitating addition of buffers to extenders (Liu *et al.,* 2016). Viability of goat semen has been better preserved if the extender has a pH range of 6.0–8.0(p*K*a of 7.0) with optimal oxygen uptake by sperms at pH 7.2 and 7.5 while optimal sperm cell motility at pH of 7.0 and 7.2 (Purdy, 2006).

Salts including sodium, citrate and citric acid together with antimicrobials like penicillin, streptomycin, lincomycin and sulphanilamide (to suppress growth of harmful micro-organism) are routinely included in semen extenders (Evans and Maxwell, 1987).

Extensive research has been carried out on extenders for their suitability in preservation of goat semen. Of these, non-fat dried skim milk extender (Corteel, 1974) or a Tris–glucose extender (Salamon and Ritar, 1982) has commonly been used for cryopreserving goat sperm and their modifications have yielded varying results (Blash *et al*., 2000).The ever-present question is whether there are optimum extenders for goat sperm or if there is a preferred one (Purdy, 2006). In bid to answer this question the specifics of constituents of extenders that take care of the requirements for goat semen preservation have been studied (Purdy, 2006).

2.5 Interactivity of goat seminal plasma with egg yolk

As already mentioned, egg yolk is a common component of semen extenders which provides protection of the sperm cell plasma membrane and has been used as Tris–egg yolk–glucose for preservation of goat semen (Purdy, 2006). However, processing goat semen using extenders containing egg yolk has resulted in variable results. In most instances the quality of the processed semen has been compromised. This has often been attributed to a known fact that seminal plasma has detrimental effect on the viability of goat spermatozoa during storage when extenders contain egg yolk (Sariözkan *et al*., 2010) due to a bulbourethral tricylglycerol lipase named Egg Yolk Coagulating Enzyme (EYCE) (Iritani and Nishikawa, 1961; Leboeuf *et al.,* 2000). EYCE acts as a catalyst that causes hydrolysis of egg yolk lecithin into fatty acids and lysolecithin (Iritani and Nishikawa, 1963). This hydrolysis induces the acrosomal reactivity by making the plasma membrane fusogenic (Upreti*et al*., 1999) in addition to causing decondensation of nuclear chromatin (Sawyer and Brown, 1995). These ultimately result in death of the spermatozoa during storage. Thus, in spite of many known benefits of egg yolk as a component of cryoprotection extenders its use in goat semen preservation suffers this setback (Drobnis *et al*., 1980; Ritar and Salamon, 1982; Memon *et al*., 1985). However, other studies found no difference in quality of semen extended with seminal plasma intact and with seminal plasma removed (Peterson *et al*., 2007; Jiménez-Rabadán *et al*., 2012).

2.6 Interactivity of goat seminal plasma with milk

Nunes *et al.,* (1982) identified a protein (SBUIII) from the goat's bulbo-urethral secretion that interacted with the constituents of a milk-based extender thereby suppressing the survival of spermatozoa. The addition of the SBUIII to washed sperm cells reduced their viability in milk-based extender, whereas the protein had no effect on spermatozoa extended in Krebs–

Ringer-Phosphate-Glucose solution. These results suggested that either the enzyme would act on milk, or conversely an enzyme from milk would act on an SBUIII component to give a product toxic to the spermatozoa. However, Courtens *et al.,* (1984) observed that vesicular secretion had protective effect against the toxic effects of SBUIII. The SBUIII component responsible for deterioration of spermatozoa extended in skimmed milk has since been identified as a monomeric 55–60 kDa *N*-glycosyl-protein (BUSgp60) that exhibits heparin affinity (Pellicer-Rubio *et al.*, 1997). In addition, BUSgp60 was found to display triacylglycerol hydrolase activity similar to pancreatic lipase-related protein 2 (PLRP2) (Giller *et al*., 1992).

The investigations suggest that EYCE and BUSgp60 lipase could be identical compounds. Both egg yolk and milk, which are otherwise considered important components of extenders, in general seem to have negative effect with goat semen. The question that remains is, what next?

2.7 Interventions to seminal plasma interactivity

The conventional method of overcoming the harmful interactivity of seminal plasma and egg yolk or milk proteins is to extend the goat semen in a buffered extender and then separate the seminal plasma from the sperm by centrifugation (Purdy, 2006; Leboeuf *et al.*, 2000; Kozdrowski *et al*., 2007). The extender or any other isotonic solution that will not kill spermatozoa can be used to wash cells either once or twice, through centrifugation and supernatant aspirated. Centrifugation is done for 10–15 min at 550–950×*g* (Leboeuf *et al*., 1998). Some of the washing media that have been used include Kreb's phosphate Ringer, normal saline, lactate ringers, or Tris based solution. Though a tedious and time-consuming process, washing semen of samples if done properly can improve semen viability (Purdy,

2006). High centrifugation revolution for a short duration has been recommended (Azerˆedo *et al.*, 2001; Burcu *et al.*, 2009; Soe Win Naing *et al*., 2010).

The absolute benefit of seminal plasma removal has however been in doubt. Certain researches indicate that removing seminal plasma is necessary for maximizing post-thaw motility and acrosomal integrity in goat semen (Drobnis *et al*., 1980; Ritar and Salamon, 1982; Memon *et al*., 1985), while others have reported positive results for sperm frozen without washing (Ritar and Salamon, 1982; Azerˆedo *et al*., 2001). Further, some studies have recorded no effects on the quality of liquid stored or frozen thawed goat semen (Peterson *et al*., 2007; Jiménez-Rabadán *et al*., 2012) while others yet recorded unfavourable results when seminal plasma was removed (Tuli and Holtz, 1994; Cabrera *et al*., 2005). The question then remains; is washing goat semen prior to extending it with egg yolk or milk beneficial or not?

Alternative extenders that minimize the sperm and lipase been proposed, including adding BUSgp60 lipase inhibitors, using lipid-free cow milk, a triglyceride-free extender containing the milk protein casein, or using milk from species other than dairy cows where fatty acid and triacylglycerol structure differ so that the enzymatic reactivity do not occur (Pellicer-Rubio and Combarnous, 1998). Kundu *et al*. (2000, 2001, and 2002) demonstrated that goat sperm can be cryopreserved in egg yolk and milk-free media, but this work was performed on cauda epididymal sperm, not ejaculated cells. This is logical because sperm from the cauda epididymis has no addition of bulbourethral gland secretion. In practice it may be tedious collecting semen using this method for commercial processing. It is rather obvious that the process could be penetrating causing pain to the buck and chances of infection are also high.

Other studies have considered incorporation of acetic and caproic acids in extenders (Leboeuf *et al.,* 2000) to maintain viability of buck semen, reduction of concentration of egg yolk (Bispo *et al.,* 2011; Beltran *et al.,* 2013) and substitution of egg yolk with synthetic compounds for example Equipro for use in ewes (Kubovičová, 2010). Soybean lecithin-based extender has equally been used as a substitute for use in goat (Roof *et al*., 2012), mountain gazelle (Saragusty *et al*., 2006), sheep (Fukui *et al*., 2008) and bovine (Aires *et al*., 2003).

2.8 Use of non-egg yolk-based extenders

Egg yolk as the main component of semen commercial extenders has been in use for decades. The other alternative has been the use of skim milk. As pointed earlier these two interfere with survival of spermatozoa of goat semen especially in presence of seminal plasma. Many research trials to have been done to formulate extenders that are free from egg yolk and milk. Soybean lecithin extenders like Bioxell® and OPTIXcell™ are commercially available and recommended for use in extension of goat semen (Vidal *et al.,* 2013). Lecithin present in egg yolk and milk is believed to restore phospholipids lost from the plasma membrane during heat shock and soybean lecithin has been shown to perform a similar function (Futino *et al*., 2010). The ability of soybean lecithin to restore plasma membrane integrity for deep-freezing of goat semen was demonstrated by Vidal *et al*., (2013) using Bioxell®.

In a study that tested the use of various fruit pulp juice to reduce the proportion of egg yolk, used to as low as 25% with bull semen could be an eye opener for trials with goat semen (Bayemi *et al*., 2015). In this study pawpaw juice, coconut milk, tomato juice and fresh Raffia Palm sap maintained acceptable progressive motility values of experimental chilled bull semen but not without incorporation of some quantity of egg yolk. Whether extenders with such fruit bases are in commercial use, is not known to the researcher. In the recent past

several research trials have been carried out to test the possibility of using coconut water based extender for extending semen from different animal species.

2.9 Coconut water as a component of semen extender

As a substitute to cow milk and egg yolk as main components of extenders, the ability of coconut (*Cocos nucifera* - a natural compound) products, to preserve semen, have been tested in various studies. For instance, coconut water used for cryopreservation of goat (Daramola *et al.,* 2016a) and boar (Luzardo *et al.,* 2010) semen; coconut milk for preservation of goat (Sule *et al*., 2007; Daramola *et al.,* 2016b) and buffalo (Rajamhendran *et al*., 1981) semen at room temperature; and powdered coconut water for cryopreservation of dog semen (Cardoso *et al*., 2005).

Coconut water has also been added to refrigeration and freezing media in sheep, swine, and bees (Kotzias-Bandeira *et al*., 1999; Almeida and Soares, 2002; Gutierrez *et al*., 2006; Nejat *et al*., 2009). These studies considered the advantage of the presence of sugars, amino acids, minerals and vitamins which are not only nutrients but also cryoprotectors found in coconut water, in addition to presence of antioxidants (Yong *et al*., 2009). The cryoprotective role, maintenance of osmotic balance and energy provision of sugars in support of survival of spermatozoa during deep freezing has widely been demonstrated (Koshimoto and Mazur,2002; Aboagla and Terada, 2003; Yancey, 2005; Naing *et al*., 2010).The total sugar content in 100mL of fresh coconut water is 6.6g of which 2.7g is glucose; 2.5g is sucrose and 2.4g being fructose (Saxelby, 2013).The amino acid component of coconut water protects the spermatozoa plasmalema from temperature related injury (Yong *et al*., 2009) through coating of the plasma membrane and combining with phospholipids on the plasma membrane (Atessahin *et al*., 2008). Viability of spermatozoa in extenders containing coconut water has also been associated to its rich content of potassium (Mansour *et al*., 2002; Yong *et al.,*

2009). Vitamin C (antioxidant) present in coconut water if supplemented with pyridoxine has been shown to reduce oxidative stress to sperm cells taking advantage of their low toxicity and water solubility (Shen *et al.,* 2010; Daramola *et al.*, 2016b). Despite these benefits, other studies reported detrimental effects over frozen-thawed sperm viability when adding coconut water to extenders meant for deep freezing storage (Santillana and Garcia, 2000; Gutierrez *et al*., 2006), possibly as a consequence of the Osmolarity change in the freezing media. However, further research on coconut water can be done to improve on making it a suitable component of goat semen preservation medium basing on the many benefits. African coastal countries like Kenya can take advantage of this to grow coconut for commercial production of coconut water. Comparison of coconut water containing semen extension media with commercially available media and the effect of seminal plasma on viability of stored goat semen is a contributing study to such endeavours.

CHAPTER3.0: MATERIALS AND METHODS

3.1 Study design

This was a cohort (longitudinal) study where semen samples collected serially (three times) from same goats were treated differently (extender, storage temperature and with or without seminal plasma) were observed for a predetermined period and the impact of the treatments on viability of spermatozoa evaluated. The effect two extenders; coconut egg yolk-based extender (COC) and commercially available extender - OPTIXcell™ (OPT), on viability of spermatozoa was evaluated in presence and absence of seminal plasma under different storage temperatures (room temperature (21-23^{0}C), chilled (1-4^{0}C) and deep-frozen in liquid nitrogen (-196^{0}C)). Viability parameters included individual progressive motility and percentage live spermatozoa assessed on daily basis for both room temperature (RT) and chilled (CH) semen. Post-thaw viability of frozen semen was tested once after being frozen for 120 days.

The ability of coconut water-egg-yolk-based extender (COC) to maintain semen viability during storage was compared against a commercially available semen extender (OPT). Two parallel experiments were done on split ejaculates, one with semen containing the seminal plasma and another with semen whose seminal plasma had been removed via centrifugation and aspiration of supernatant. Both experiments were run simultaneously utilizing each half aliquot of semen collected from the same buck on the same day. Three serial collections of ejaculates were obtained from every experimental buck three days apart except buck5 from which only second trial of ejaculate collection was successful.

3.2 Buck recruitment and care

Five mature (2-4 years old, weighing between 40-56kg live body weight), Alpine bucks were randomly selected out of 10 bucks in a buck stud located at the Animal Health and Industry

Training Institute (AHITI)-Ndomba - Kenya. The 10 bucks were housed in individual pens without following any order. The bucks were numbered from one to ten and random numbers were generated from a scientific calculator (FX-82MS- CASIO) to select five bucks.

The bucks were already familiar with the artificial vagina method of semen collection. They were re-evaluated for breeding soundness based on standard procedures (Mushtaq *et al*., 2007), then transported by vehicle to the University of Nairobi (UoN) Animal unit at the Department of Clinical Studies. They were treated for external and internal parasites using 1% ivermectin (supermec® Bimeda ltd. Nairobi, Kenya) injected subcutaneously at 0.2mg/kg body weight. At the animal unit, the bucks were kept in pairs in cubicles except one buck (buck 1), which was kept on its own. Within the cubicles, the bucks were tethered far from each other to avoid fights. The goats were allowed *adlibitum* access to good quality grass hay and clean water. In addition, each goat was given 1kg of a commercially available concentrate mixture (dairy meal; Pembe Millers Ltd, Kenya) daily divided in two meals.

Daily health check was done on all the goats. This included daily visual clinical examination, auscultation of various body regions as well as recording their body vital parameters each morning.

The bucks were allowed two weeks of acclimatization, after which two semen collections were obtained from them to re-affirm their maintenance of quality semen production in the new environment. To augment this, twice-weekly testicular palpation and ultrasonography was performed on them during which one of the bucks (buck 2) was withdrawn from the study after he developed orchitis thus leaving four bucks for the experiments.

3.3 Semen collection and initial evaluation

This study was conducted in the month of February - April 2015. From the third week, semen was collected from each buck using the standard artificial vagina (AV) technique (Jiménez-Rabadán *et al*., 2012) after every three days as from 0900hrs on each collection day, so as to obtain a total of three ejaculates per buck for the experiments. In brief, the AV had an insulated outer casing (15cmx5.5cm) and an inner liner made of thin rubber. In assembling the AV, the liner was extended at least 2-3cm beyond the end of the outer casing and folded back and secured with rubber bands to form a water-tight jacket. The jacket was then filled with water (at 50^0C) through a tap on the side of the AV, to about two-thirds to achieve the recommended 45^0C inside the AV. The end of the AV through which the penis would be introduced was lubricated with a non-spermicidal gel (#xB640, L'aigle Cedex, France). At the other end of the AV, a plastic cone with a calibrated glass tube was fixed and the glass tube leather cover closed. Figure 3.1 below is a diagram showing different part of an artificial vagina.

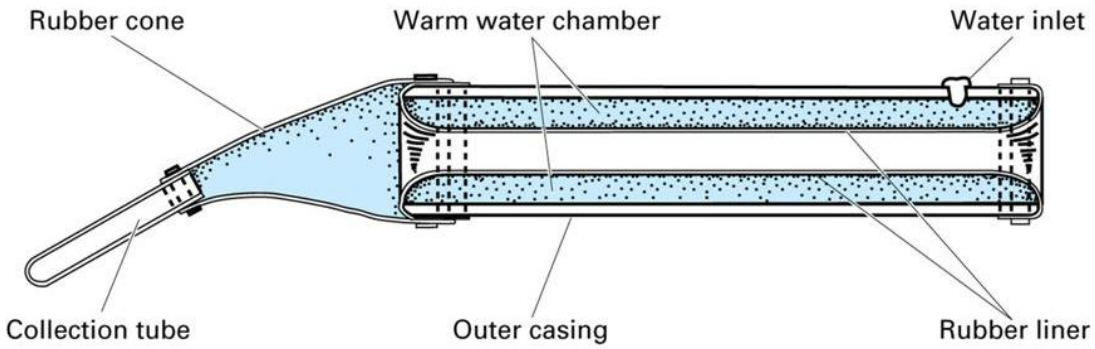


Figure 3.1 Longitudinal section of an artificial vagina.
Source: http://slideplayer.com/slide/4404429/

The prepuce of the buck was wiped with a clean paper towel to reduce contaminants at the prepuce. A teaser doe was immobilized in a standing position from her head end by an assistant exposing her rear end. The assistant held firmly a rope tied to the neck of the doe with the right hand. The left hand was used to secure the head between the hand and the waist of the assistant. This ensured minimal movement of the doe. The operator crouched at the right of the doe and held a fully assembled AV with the right hand along her flank with the open end facing downwards at an angle of 45^0towards the male held on a leash by another person and led toward the female. The male was allowed two false mounts but finally allowed to mount the doe. The penis was directed by holding the prepucial skin with the left hand to the open end of the AV. An upward forward thrust with an upward jerking of the head by the buck was an indication that ejaculation may have occurred. The buck was then allowed to dismount. The graduated tube (covered in a leather covering) containing the ejaculate was separated from the cone and capped with aluminum foil then labeled with the buck's identity. The tube was removed from the covering and immediately placed in a water bath at 37^0C. Only one ejaculate was obtained from each individual buck on a given day of semen collection. Once collected, routine evaluation for colour, volume, consistency and admixtures was done (Shamsuddin *et al.,* 2000).

Semen was also evaluated for mass activity on a scale of + to ++++ (Mushtaq *et al.,* 2007). Mass activity was determined by placing a drop of raw semen on a warm slide on a heated microscope stage at 37^0C and examined at x10 objective lens. If only individual cells were observed moving then a single plus sign (+) would be assigned. Islands of individual cells plus slow waves would be represented by (++), vigorous mass activity was indicated by (+++) and turbulent mass activity was assigned (++++). In this study, numerical value scaling of 1-4 was awarded in the same order for purposes of statistical analysis.

Progressive forward motility (Burcu *et al*., 2009; Soe Win Niang *et al.*, 2011) and membrane integrity, using eosin-nigrosin staining technique (Kulaksiz *et al*., 2013) were also evaluated. To assess progressive forward motility, a drop of semen was mixed with a drop of warm 0.9% saline (Claris Otsuka Private Limited- village-Vasana- Chacharwadi India) on a warm microscope slide to achieve a 1/1 v/v extension. The drop was covered with a cover slip and motility determined through visual observation of 8-10 separate fields under a warm-stage light microscope at x400 (oil emulsion lens) by one experienced person throughout this study. Percentage individual progressive motility was subjectively determined by counting ten spermatozoa and indicating how many of them moved across each microscopic field and the totals averaged to determine the percentage of motility score. This was repeated for all bucks on each day of semen collection. Values obtained, (mean±SD) formed the baseline progressive motility for the ejaculates from each buck for the day of semen collection.

To assess plasma membrane integrity, a drop of semen was placed on a warm glass-slide at 37^0C side by side with warm eosin-nigrosin stain and the two allowed to mix for 15-60 seconds by gently tilting the slide back and forth. A thin smear was then prepared, air dried and observed under microscope at x400 to determine live/dead ratio. Nigrosin provided a dark background while eosin stained the cytoplasm of the spermatozoa pink if the plasma membrane was broken. Sperm cells showing partial or complete colorization were considerednon-viable or dead (Kulaksiz *et al.,* 2013). Cells that did not pick stain were considered viable. A minimum of two hundred sperm cells were counted from several microscope fields. All cells on the last field were counted even if the 200 cells target had been reached. Viable spermatozoa were counted against non-viable ones and percentage live (viable, non-stained) cells determined.

Ejaculate concentration was determined using a spectrophotometric machine (Acucell^{TM}-IMV technologies), as per the directions provided (www.imv-technologies.com), after a pre-test showed that such concentrations of the experimental buck semen fell within the normal range for goats. Three thousand nine hundred and sixty micro litres (3960µL) of 10% sterile sodium chloride solution at 37^0C (water bath) was transferred into a sterile cuvette using a micropipette. Forty micro litres (40µL) of semen was then added to the saline to top up the volume to 4000µL and by gentle mixing, the spermatozoa allowed to distribute evenly in it. The cuvette was then immediately inserted into its slot in the spectrophotometer and ejaculate volume fed into the machine. The machine was then able to display the total sperm content of the ejaculate in billions per millilitre. The machine set for an extension rate of 40million spermatozoa per millilitre for purposes of this study. The machine was then able to generate the volume of extender to be added to the ejaculate.

3.4 Semen extender preparation

Two extenders were used in the current study; coconut water based preparation (COC) and a commercially available extender called OPTIXcell™ (OPT).

3.4.1 Coconut water extender

Coconut fruits were bought from the market, brought into the laboratory, and selected. Selection was based on presence of coconut water determined by shaking the fruit. Greener fruits were selected because they were considered fresh. The fruits were washed with clean water, rinsed, and dried with disposable paper towel. They were routinely opened to access the water in them. The coconut water was obtained by aspiration using sterile rubber capped pipette from each fruit in separate 100mL beakers. Each sample was taken through organoleptic evaluation by one staff from the Kenya Animal Genetic Resource Centre (K.A.G.R.C) who had the experience of relating taste to coconut water quality. A sample was

accepted for use if it had a strong sour taste. Selected coconut water samples were then pooled together and used for preparing the final extender.

To obtain egg yolk, fresh non-fertilized chicken eggs (less than seven days old) were obtained from the poultry unit at the Animal Production Department, University of Nairobi on every extender preparation day. The eggs were wiped using paper towels to clean off particulate dirt. The surfaces of the eggs were further wiped with a cloth towel damped in distilled water and allowed to dry. The eggshell was carefully broken across the longitudinal axis of the eggs. The content of the eggs were gently emptied onto a sterile absorbent paper placed over a sterile paper foil care being taken not to break the egg yolk membrane. The albumen was removed by gently rolling the contents from each egg from one absorbent paper to the other. The egg yolk membrane was then carefully punctured and the egg yolk drained into a sterile beaker while ensuring the membrane was held back in the paper.

The antimicrobials used in this study included sulphanilamide (1.2g) (Loba Chemie-India), streptomycin (0.54g) (CSPC Group pharmaceuticals-China) and penicillin (0.24g) (CSPC Group pharmaceuticals-China). Penicillin, streptomycin, and Sodium citrate (Manigate agencies) were weighed and put in one flask while sulphanilamide (1.2g) was weighed and placed in a different flask.

Twenty eight millilitres (28mL) of egg yolk was measured into a third flask. 100mL of double distilled water was added to the flask containing sulphanilamide and another 100mL of the water added to the flask containing the other mixture. Each of the flasks was warmed in a larger container containing hot water and shaken to allow the solutes to dissolve. The contents of the flasks were then mixed in one flask and allowed to cool to room temperature by placing the flask in water at room temperature and the temperature of its content

monitored. Separately, coconut water was boiled for 15minutes, sieved, and allowed to cool to room temperature by placing the flask in water at room temperature as well. Sieving was done by placing sterile double folded gauze in a glass funnel and the coconut water transferred into a different beaker via a funnel. Sixty-eight (68mL) of the coconut water was then added to the flask with the dissolved solutes. Twenty-eight millilitres (28mL) egg yolk was then added to the miture. The mixture was mixed by gently tilting the flask back and forth. Double distilled water was then added to the mixture at room temperature to top it up to 400mL. The temperature of the mixture was raised to 37^0C by placing the flask in water bath at the same temperature while monitoring the temperature. The pH was read using a pH metre (Hanna instruments) and adjusted to between 7.3-7.5 using 10% sodium hydroxide (Manigate agencies) at 37^0C. Fresh extender was made on each day of semen collection and the extender used within six hours of preparation. Figure 3.2 below summarizes the process of preparation of COC extender.

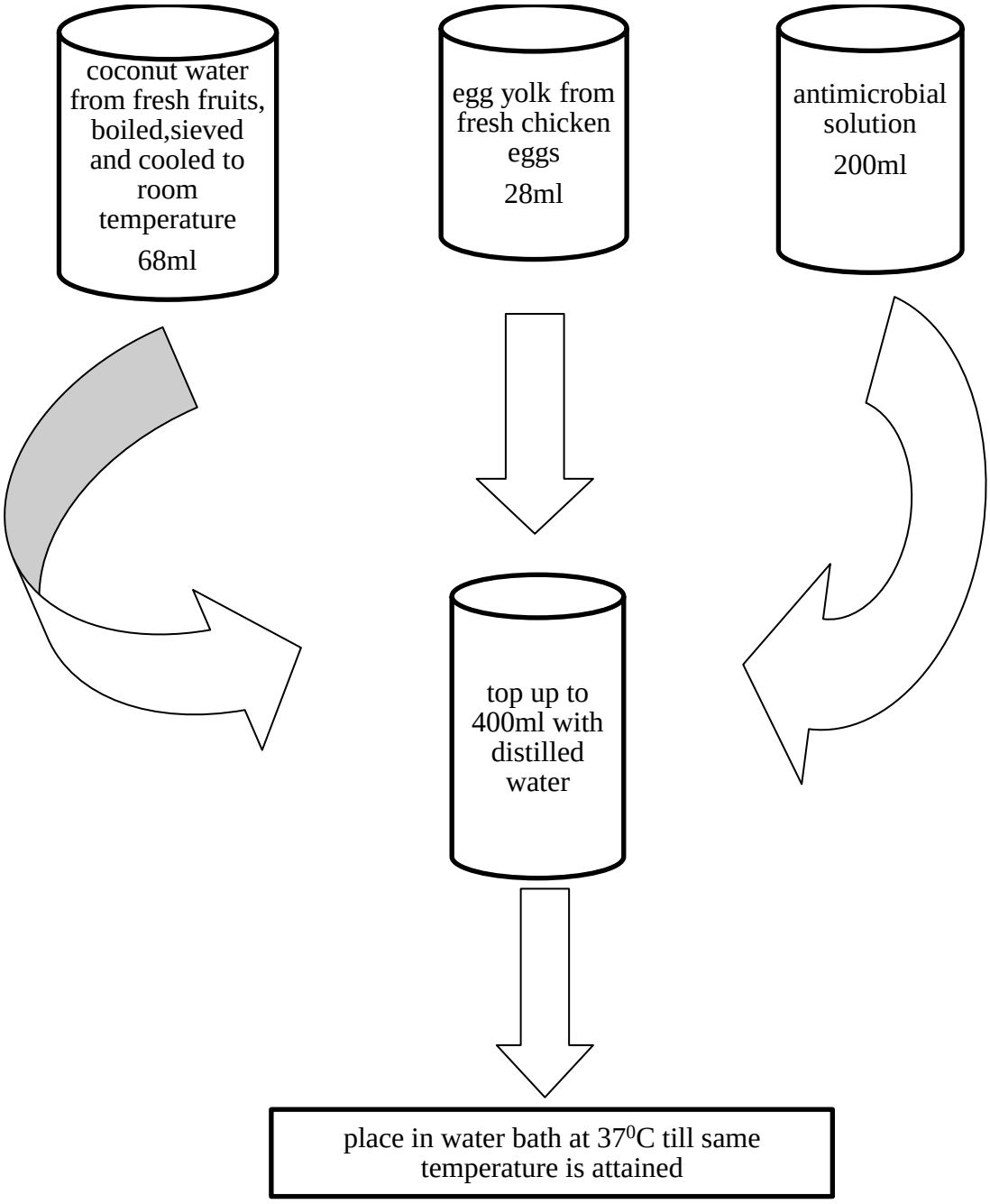


Figure 3.2 Flow chart showing summary for preparation of COC extender

3.4.2 OPTIXcell™ extender

For purposes of this study, OPTIXcell™ was identified as OPT. It was a commercially available extender made without any animal protein as indicated by the manufacturers (IMV

Technologies – www.imv-technologies.com). It was prepared for use as per the manufacturer's instructions. One hundred and fifty millilitres (150mL) of the extender was reconstituted by mixing 50mL of OPT with twice the amount (100mL) of double-distilled de-ionized water at 37^0C. To achieve this, the capped 50ml bottle of OPT was placed in water bath for 10 minutes. One hundred (100mL) of double distilled water was put in a flask and the flask placed in the same water bath at 37^oC for 10 minutes to equilibrate their temperatures. The bottle of OPT was removed from water bath, wiped dry using a paper towel and its content poured into the flask containing distilled water and the bottle rinsed with the same solution. The fresh extender was kept in the same water bath and used within six hours.

3.5 Sample aliquots

Each ejaculate was divided into two equal aliquots using micropipette, one for experiments with seminal plasma (A) and the other for experiments without seminal plasma (B). Each of the aliquots (A and B) was divided further into two aliquots for extension with two extenders, COC and OPT. The aliquots were kept in centrifuge tubes placed in water bath at 37^0C throughout the processing.

Aliquots of semen meant for experiments without removal of seminal plasma identified as 'A' samples were added to appropriate volumes of extenders at 37^0C. The extended portions were split into three equal portions to be used for storage at room temperature (RT) semen, at cold room temperature as (CH) semen and the other to be frozen and stored in liquid nitrogen as deep-frozen (DF) semen. Figure 3.3 below summarizes the processes of treatment of ejaculates collected.

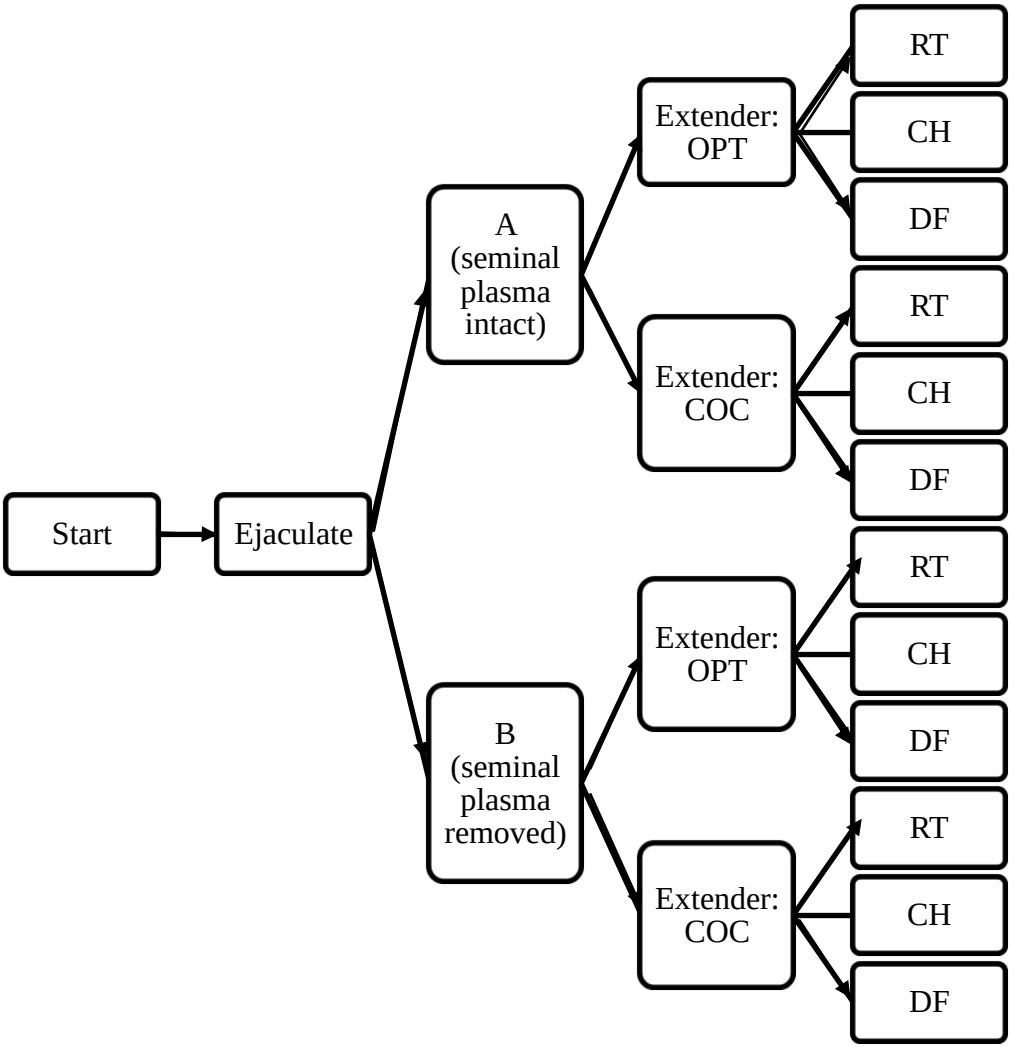


Figure 3.3 Flow chart showing extension and storage method used for buck semen
COC= coconut water based extender, OPT= OPTIXcell™ (commercial extender), RT= room temperature semen storage, CH= chilled semen storage, DF= deep-frozen semen storage.

3.6 Removal of seminal plasma

Volumes of aliquots B were measured using micropipette and recorded. The aliquots were added to 5mL of 0.9% of a commercially available normal saline (Claris Otsuka Private Ltd - India) in capped centrifuge tubes at 37^0C and seminal plasma separated from the cells through

centrifugation at 3000 revolutions per minute (rpm) for 10mins at 37^0C and the supernatant carefully aspirated using a glass pipette with a rubber bulb at one end.

The pellet remaining at the bottom of the centrifuge tube was reconstituted to the original volume of aliquot B using normal saline at 37^0C.The centrifuge tube was then gently tilted back and forth until the entire pellet was re-suspended then placed again in water bath ready for addition of extender. Each extender (at 37^0C) was then gently added to respective seminal plasma-free semen sample portions at the appropriate volumes. Each of the extended samples was further split into three equal portions and placed in water bath ready for storage at room temperature, cold room or to be deep-frozen (see the freezing method in section 3.10). Figure 3.4 below shows extended semen samples in centrifuge tubes prior to centrifugation while figure 3.5 shows the pellets of spermatozoa after centrifugation and removal of the seminal plasma.

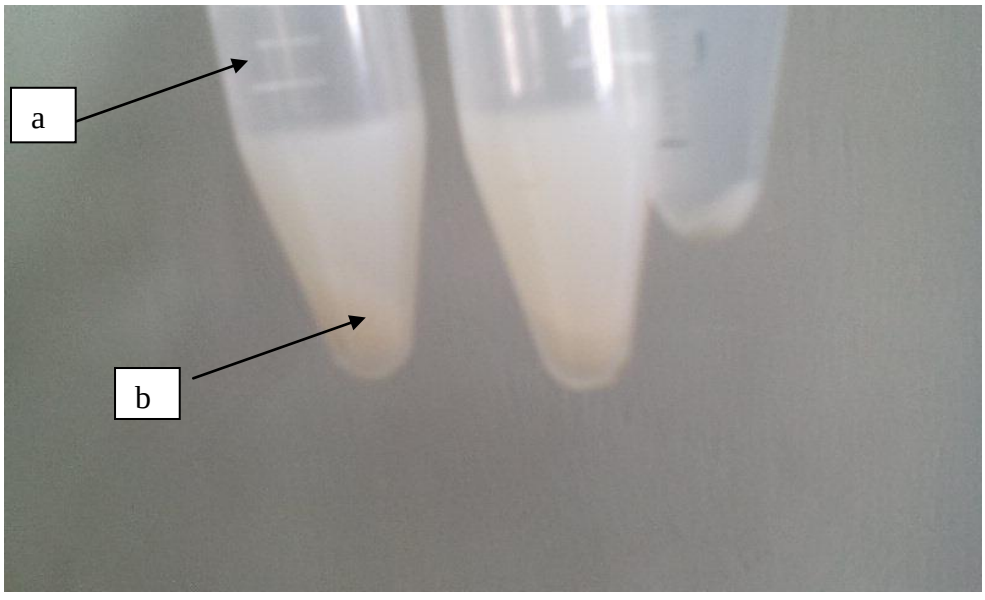


Figure 3.4 Three semen samples extended in normal saline prior to centrifugation
(a) Graduated centrifuge tube; (b) suspension of semen in normal saline

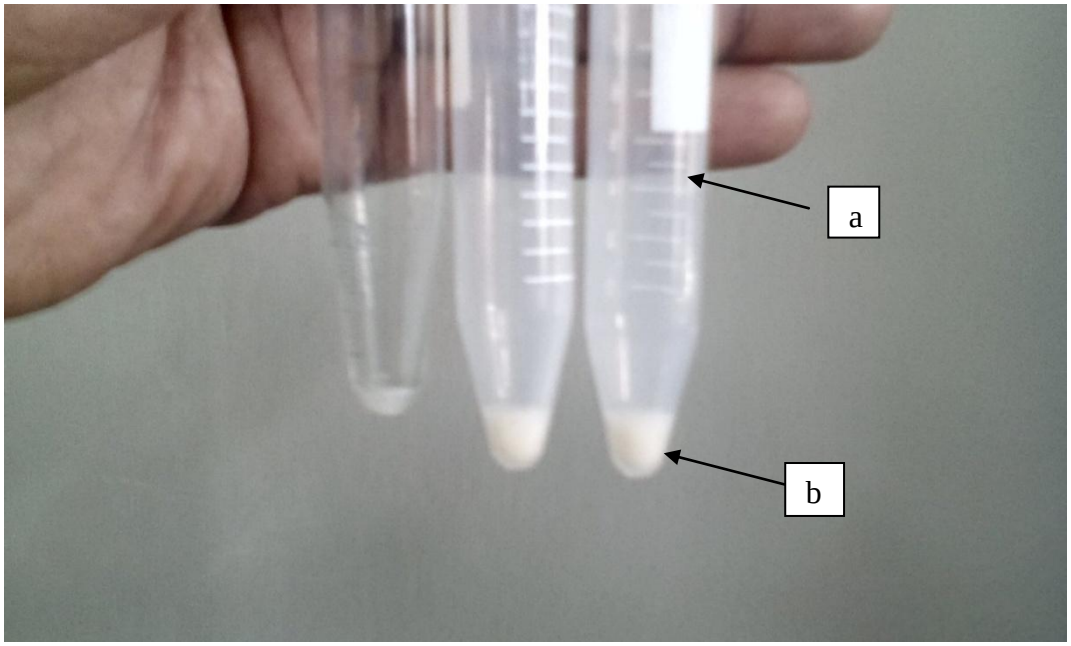


Figure 3.5 Three semen pellets samples after removal of seminal plasma
(a) Graduated centrifuge tube; (b) semen pellet

3.7 Room temperature (RT) storage

Centrifuge tubes with semen destined for room temperature storage were kept in water bath and power to the water bath machine switched off and the temperature of the water monitored to drop to room temperature using an alcohol thermometer placed in the water bath. The samples were then removed from the water bath, placed in a rack and kept in a lockable shelf away from direct light.

3.8 Chilled (CH) semen processing and storage

Semen samples meant for cold room storage (1-4^{0}C) were placed in water collected from the water bath at 37^{0}C, placed in a cool box with ice packs, then transported by vehicle to the cold room located approximately three kilometres away from the processing point. Bath

water was allowed to cool to cold room temperature. Samples were then removed from the bath water placed in racks and kept on a bench in the cold room.

3.9 Deep freezing (DF) storage

Samples for deep freezing were treated as described for chilled semen and prepared for packaging in pvc straws. All the packaging equipment used were all along kept in the cold room to attain the cold room temperature. The samples were gently mixed to allow spermatozoa to be equally distributed. The samples were then removed from water then packaged in 0.25mL labelled mini-straws, sealed with polyvinyl chloride (PVC) powder at the open ends of the straws, and the straws placed in a container with water at 4^0C for 30mins,to allow the powder seal to absorb water and set. The straws were then arranged on a rack and left in the cold room for between 1-2 hours to allow equilibration. The rack was then placed in liquid nitrogen vapour (4cm above liquid nitrogen) for seven minutes before all the straws were collected using a pair of tongs (previously kept in the liquid nitrogen vapour), plunged into a goblet containing liquid nitrogen which was then dipped into large storage tank of liquid nitrogen.

3.10 Semen viability evaluations

Parameters for semen viability used in this study included separate daily assessment of individual progressive motility herein referred to as motility, and percentage live spermatozoa for semen stored at room temperature and in the cold room until the values for individual samples recorded zero. Deep-frozen samples were analyzed only once for similar parameters after 120 days in liquid nitrogen.

Motility was assessed by placing a drop of semen on a warm microscope slide at 37^0C for 15 seconds prior to visual observations of microscopic fields. Percentage motile spermatozoa were estimated by visual examination of the average number of spermatozoa that swam

across 8-10 observation fields, as described in section 3.4. Percentage of live spermatozoa was determined as a ratio of live spermatozoa of all cells counted for each sample (section 3.4).

3.11 Data analysis

Data were coded for analysis with Statistical Package for Social Sciences (SPSS) version 20.0. (SPSS Inc.-Chicago). The median and range of semen parameters before treatments were displayed. The values of semen parameters obtained initially after collection of semen served as the reference for comparison with post-treatment values obtained within two hours following treatment. Analysis of variation in the mean initial semen viability evaluation parameters were done to establish effect of buck and day of collection. Pre- and post-treatment median values were compared to determine effect of extension. Post-treatment mean (Mean±standard deviation (SD)) semen viability values served as reference for comparison with daily changes in parameter values. Homogeneity tests (Box's test and Levene's test) using the General Linear Model (GLM), were done to confirm that the values did not have significant variances. Box's test tested the null hypothesis that the observed covariance matrices of the dependent variables (motility and percentage live spermatozoa) were equal across groups. Levene's test tested the null hypothesis that the error variance of the dependent variables was equal across groups.

Values obtained from daily analysis were utilized to assess the trends of changes occurring due to capability and/or non-capability of the extender to maintain spermatozoa viability during storage of semen with or without seminal plasma. Kaplan-Meier survival analysis was used to demonstrate survival function of daily evaluation parameters recorded over the storage period under different treatments. The survival distributions for motile and live

spermatozoa were tested using the Log Rank test. Data were presented in tables and output line plots.

Daily changes were assessed using the GLM. Analysis was done independently for each of the dependent variables (motility and percentage live spermatozoa). Effects of the fixed factors (extenders, seminal plasma, and the interactive effect of the two (at 95% level of confidence)) on the mean values of each viability evaluation parameter at 50% cut-off for semen stored in liquid form (RT and CH semen) were tested using the GLM equation:

$$\gamma = \beta 0 + \beta 1X1 + \beta 2X2 + \beta 3X3 + e$$

Where,

$$\gamma = dependentvariable(motility\ or\ percentage\ livespermatozoa)$$

β0=the underlying constant (intercept)

*β1= rate of change (gradient) of γ due to X*1(seminal plasma)

*β2=rate of change (gradient) of γ due to X*2 (extender type)

β3=rate of change (gradient) of γ due toX3

*X3=interactive effect of X*1(seminal plasma) *and X*2 (extender type)

e= random error assumed to be normally distributed

The number of days such semen maintained viability above 50% were displayed for purposes of extrapolated practicability (days in which the semen would likely be used with potential reasonable fertility given percentage motile and live spermatozoa, insemination technique and good timing).

Analysis of variances in mean post-thaw parameter values were compared for deep-frozen semen against each seminal plasma status and extender type at 95% confidence level.

CHAPTER4.0: RESULTS

4.1 Sample numbers

Semen was collected from four out of the initial five bucks after one buck (buck2) developed orchitis. Ten ejaculates were collected in the three collections since one of the bucks cooperated only on one collection day. From each ejaculate, four samples were generated for each of the storage temperature modalities (room temperature, chilled and deep freezing temperatures). For room temperature and chilled semen, 40 vials of samples were generated for each of the storage modalities while deep-frozen semen yielded 458 straws each containing $40X10^6$ motile spermatozoa per mL at the beginning of the freezing process. Forty million spermatozoa per mL was chosen for purposes of the current study in order to have reasonable volumes of extended semen for storage.

4.2 Evaluation of pre-extension parameters

Parameters considered for pre-extension evaluation included ejaculate volume and colour, spermatozoa concentration, individual motility and mass activity. Analysis of variance of means of the parameter values was done and effects of bucks and day of collection compared. The results were presented as shown in Tables 4.1- 4.3 below.

Table 4.1 Evaluation pre-extension parameters of buck semen (n=4)

Parameter	Day	Buck ID				
		1	3	4	5	**Median(max-min)**
Volume	1	0.8	0.9	1.1		**0.9(0.8-1.1)**
	2	0.4	1.1	0.5	0.4	**0.45(0.4-1.1)**
	3	0.5	1.2	1.4		**1.2(0.5-1.4)**
Colour	1	Creamy	Creamy	Whitish creamy		
	2	Creamy	Creamy	Whitish creamy	Creamy	
	3	Creamy	Creamy	Whitish creamy		
Mass activity	1	4	3	3		**3 (3-4)**
	2	2	3	2	4	**2.5(2-4)**
	3	4	3	3		**3(3-4)**
Concentration(million cells/mL)	1	3308.0	3103.0	2958.0		**3103(2958-3308)**
	2	2863.0	3437.0	2619.0	3588.0	**3150(2619-3588**
	3	4080.0	3420.0	3310.0		**3420(3310-4080)**
Motility (%)	1	95.0	85.0	90.0		**90(85-95)**
	2	90.0	95.0	80.0	90.0	**90(80-95)**
	3	90.0	90.0	90.0		**90.0**
Live (%)	1	100.0	97.4	99.1		**97.4(99.1-100)**
	2	95.1	100.0	96.2	97.1	**96.7(95.1-100)**
	3	97.8	97.1	100.0		**97.1(97.1-100)**

From table 4.1 above, the ejaculate volume from the bucks ranged from 0.4-1.4 millilitres (mL). The colour of ejaculates was either creamy or whitish creamy. The mass activity of the ejaculates of all the bucks ranged from 2-4, measured in a scale of 1-4. The concentration of all ejaculates (millions/millilitre) ranged between 2619-4080. The percentage individual spermatozoa progressive motility of all ejaculates ranged from 80-95%. The mean of percentage live spermatozoa ranged from 95.1-100%.

Table 4.2 Effects of buck (n=4) and day of semen collection on semen parameters

Parameter		F	Sig.
Volume	Buck	0.2	0.54
	Day	1.5	0.28
Concentration	Buck	0.9	0.51
	Day	1.6	0.27
Motility (%)	Buck	0.4	0.74
	Day	0.09	0.92
% Live	Buck	0.06	0.98
	Day	0.5	0.66
Mass activity	Buck	8.0	0.036
	Day	2.0	0.25
Colour	Buck	2.9	0.16
	Day	1.0	0.44

F= f statistic value, Sig.= value of significance at 95% confidence level

The effect of individual buck and day of semen collection was evaluated and results presented as shown in Table 4.2 above. Individual buck or day of semen collection did not have any significant effect on ejaculate volume, spermatozoa concentration, percentage motile spermatozoa, percentage live spermatozoa and ejaculate colour ($p>0.05$). However, buck effect on the mean mass activity was significant ($p<0.05$). Day of semen collection did not have any significant effect on the mass activity of the ejaculates collected ($p>0.05$).

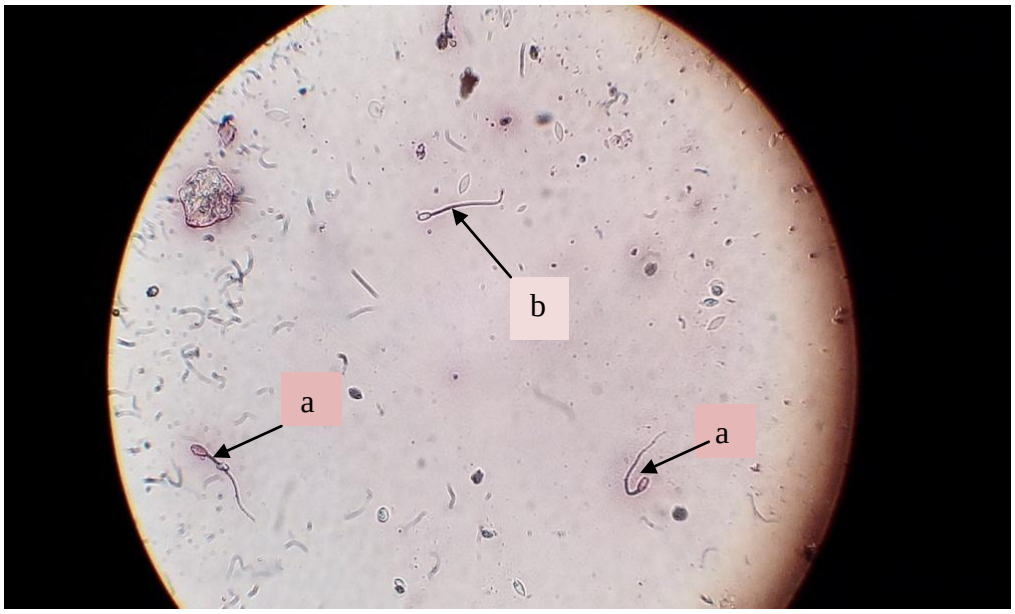


Figure 4.1 Microscopic field showing live and dead sperm cells
Two dead sperm cells (a; with pink stained heads) and one live cell (b; head not stained)

4.3 Analysis comparing evaluation parameters before and after extension

Mean values for motility and percentage live spermatozoa were compared before and after semen samples were extended with specific extenders, with and without removal of seminal plasma, using t- test statistic. The results of these analyses were as presented in the Table 4.3 below.

Table 4.3 Comparison of significance of pre- and post-extension of means of evaluation parameters

Parameter	Treatment	N	Median %	Minimum %	Maximum %	P value
Motility	Pre-extension	10	90	85	100	
	Post-extension (ACOC)	10	80	70	85	0.0
	Post-extension (BCOC)	10	80	70	85	0.0
	Post-extension (AOPT)	10	80	70	90	0.004
	Post-extension (BOPT)	10	80	80	85	0.0
% live cells	Pre-extension	10	97.6	95.1	100	
	Post-extension (ACOC)	10	96.7	94.7	100	0.09
	Post-extension (BCOC)	10	93.1	84.2	98.1	0.001
	Post-extension (AOPT)	10	97.3	93.1	100	0.22
	Post-extension (BOPT)	10	94.3	87	98.1	0.005

From Table 4.3 above, extension of semen reduced the motility of the spermatozoa when seminal plasma was intact and when removed ($p<0.05$). Similarly, the percentage of live spermatozoa reduced significantly in samples where seminal plasma was removed ($p<0.05$). However, following extension samples where seminal plasma was intact did not have a significant reduction in mean percentage live spermatozoa ($p>0.05$).

4.4 Homogeneity test for viability evaluation parameters after semen extension

Homogeneity tests included descriptive statistics for post-extension viability parameter values, Box test and Levene's test as shown in Table 4.4-4.7 below.

Table 4.4 Comparison of means of viability evaluation parameters after extension of semen using two different extenders with and without seminal plasma

Descriptive Statistics					
Parameter	**Seminal Plasma**	**Type of Extender**	**Mean**	**Std. Deviation**	**N**
Percentage Motility	A	COC	79.5	3.7	10
		OPT	80.5	6.9	10
	B	COC	78.5	4.7	10
		OPT	80.5	1.6	10
Percentage Live Spermatozoa	A	COC	97.0	1.8	10
		OPT	97.3	2.3	10
	B	COC	92.8	3.9	10
		OPT	93.9	3.1	10

After semen sample treatments, overlaps of mean values as indicated by the standard deviations from the means were recorded within each group of each dependent variable (semen viability evaluation parameters) (Table 4.4 above).

Table 4.5 Box's test of equality of covariance matrices

Box's M	25.7
F	2.6
df1	9
df2	14851.9
Sig.	0.006

Tests the null hypothesis that the observed covariance matrices of the dependent variables are equal across groups.

The observed differences in mean viability evaluation parameters (groups) in Table 4.4 were significantly different ($p<0.05$) (Table 4.5) implying that recorded percentage motility values belonged to a different population from the percentage live spermatozoa values.

Table 4.6 Levene's test of equality of error variances

	F	df1	df2	Sig.
Percentage Motility	2.6	3	36	0.07
Percentage Live Spermatozoa	1.04	3	36	0.39

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

*Source: GLM equation Design: Intercept + Plasma + Extender + Plasma * Extender*

*=interaction of the variables

Leven's test of equality of error variances in mean viability values (Table 4.6 above) indicated that following treatment, values within each viability evaluation parameter group were not significantly different and therefore considered to belong to independent populations ($p>0.05$).

Table 4.7 Significance of effect of seminal plasma and/or extender on the observed differences in mean evaluation parameter values

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Observed Power
Seminal Plasma	Percentage Motility	2.5	1	2.5	0.1	0.7	0.1
	Percentage Live Spermatozoa	148.9	1	148.9	18.0	0.0	0.99
Extender	Percentage Motility	22.5	1	22.5	1.1	0.3	0.17
	Percentage Live Spermatozoa	5.2	1	5.2	0.6	0.4	0.12
Plasma* Extender	Percentage Motility	2.5	1	2.5	0.1	0.7	0.1
	Percentage Live Spermatozoa	1.4	1	1.4	0.2	0.7	0.1

*=interaction of the variables

Table 4.7 above demonstrated that seminal plasma had significant effect on the percentage of live ($p<0.05$) but not motile ($p>0.05$) spermatozoa. Both extenders did not have any significant effect on motility or percentage live spermatozoa ($p>0.05$). The interactive effect of the extenders and seminal plasma had no significant effect on either motility or percentage live spermatozoa ($p>0.05$).

4.5 Effect of storage temperature (21-23^{0}C, Room Temperature - RT and 1-4^{0}C, CH -chilled) on viability of buck semen

The range of duration from when loss of spermatozoa viability began to when the last sample lost viability under different treatments, as presented in Table 4.8. Further analysis of mean survival time (Mean±standard error of mean (sem)) for percentage motile and live spermatozoa was done using Kaplan Meier survival analysis, over the period of storage for RT and CH semen extended with and without seminal plasma. Survival time estimations for motility and percentage live spermatozoa were limited to the largest survival time if they were censored above 0%. Zero percent indicated that the event (rejection of the sample) had occurred. Significance of the differences in the survival distribution of percentage motile and live spermatozoa, were tested using Log Rank Chi-Square tests (χ^2) and the results presented in tables and plots of survival distribution curves for different categories of semen treatments. Survival distribution curves were used to display survival duration in each of the extenders with or without seminal plasma under both temperatures of storage and further comparison of survival time done at mean cumulative survival distribution (sections 4.5.1-4).

Table 4.8 Duration buck spermatozoa stayed motile and live in samples extended with and without seminal plasma, at RT and CH storages

Temperature of storage	Extender	Seminal plasma	Duration of viability (Days)	
			Motility	% live cells
RT	COC	A	3-12	4-16
		B	9-17	10-20
	OPT	A	4-15	5-17
		B	8-17	11-19
CH	COC	A	11-28	12-29
		B	13-35	14-37
	OPT	A	15-36	16-36
		B	21-33	23-36

As indicated in table 4.8 above, under each temperature of storage (RT and CH), longer durations of viability were recorded when seminal plasma was removed (B). Semen samples extended in COC with intact seminal plasma (A) were the first to lose viability. However, when seminal plasma was removed, COC recorded the longest duration of viability (37days) especial for chilled semen. Semen stored chilled recorded relatively longer duration of viability compared to room temperature storage. Semen stored at room temperature recorded individual progressive motility for up to 17 days, but showed presence of live spermatozoa for up to 20 days. Semen stored chilled remained motile for up to 36 days and showed presence of live spermatozoa for up to 37days.

4.5.1 Analysis of effect of extenders on survival time and distribution of buck semen stored at room temperature

Table 4.9 Analysis of mean survival time and test for survival time for percentage motile and live spermatozoa in different extenders for RT semen

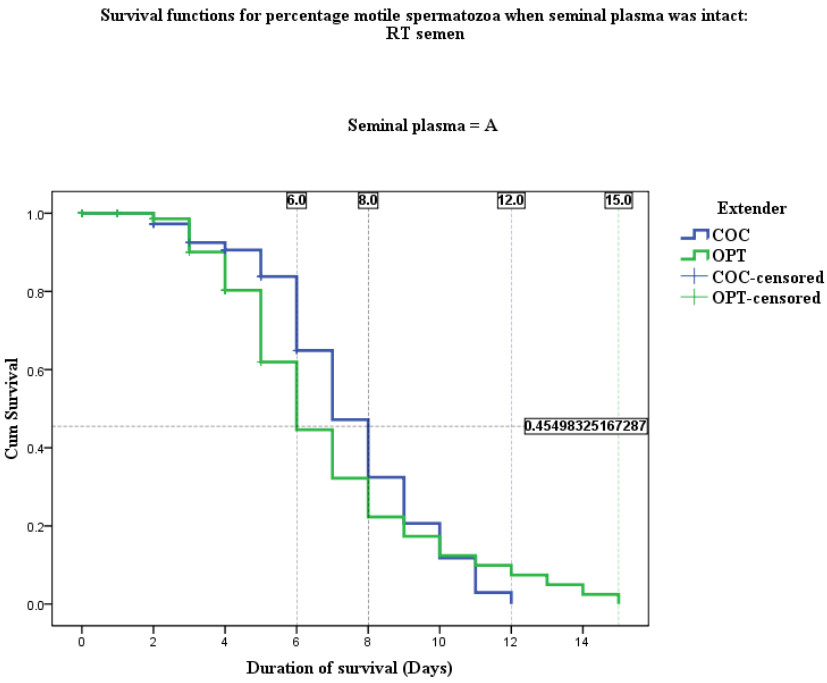
Viability parameter	Seminal plasma	Extender	Percent censored	Mean[a]		Log Rank (Mantel-Cox)			
				Estimate	Std. Error	COC χ^2	COC Sig.	OPT χ^2	OPT Sig.
Motility	A	COC	87.6%	10.05	.48			.088	.766
		OPT	88.3%	11.92	.87	.088	.766		
Percentage live	A	COC	90.4%	13.35	.73			.001	.975
		OPT	90.2%	13.83	.91	.001	.975		
Motility	B	COC	90.9%	14.66	.57			3.019	.082
		OPT	92.8%	15.81	.32	3.019	.082		
Percentage live	B	COC	93.6%	17.79	.62			.192	.662
		OPT	94.1%	17.911	.306	.192	.662		

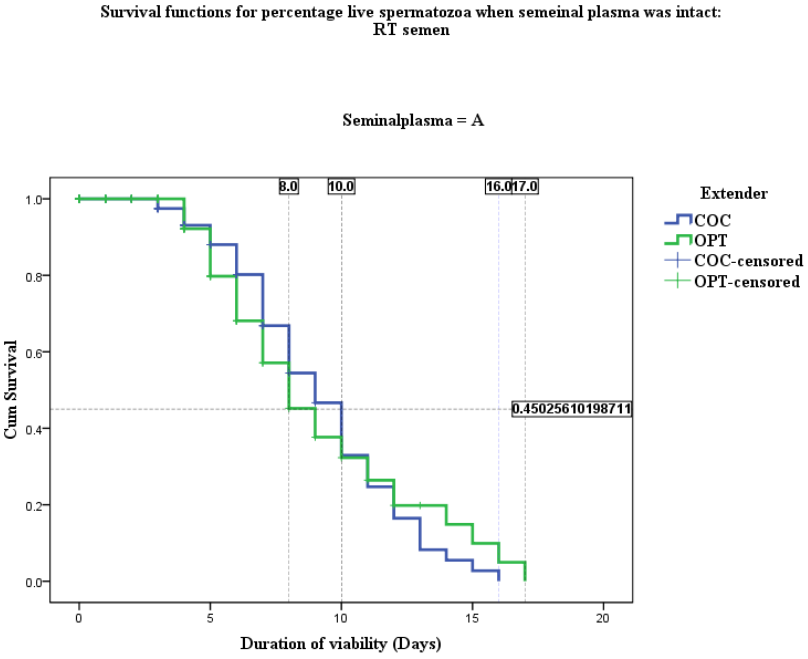
a. Estimation is limited to the largest survival time if it is censored.

Effect of extender on each viability parameter was tested under individual seminal plasma status (Table 4.9). The percentages of censored sample were generally higher for semen extended in OPT. The mean survival time of percentage motile and live spermatozoa were better when semen was extended in OPT compared to when semen was extended in COC for RT semen irrespective of seminal plasma status.

The observed differences in the longevity of spermatozoa in each of the extenders (mean survival time) had no statistical significance under similar seminal plasma status for semen samples stored at room temperature ($p>0.05$). This indicates that there was equality in survival of percentage motile spermatozoa in the two extenders when seminal plasma was intact (A) and when seminal plasma was removed (B).

(a)



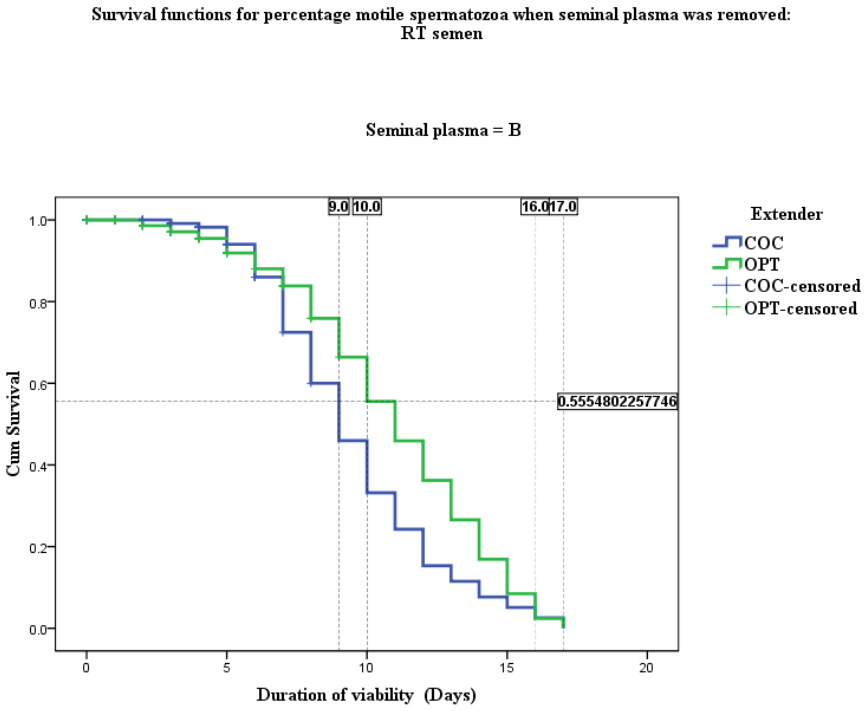


(b)

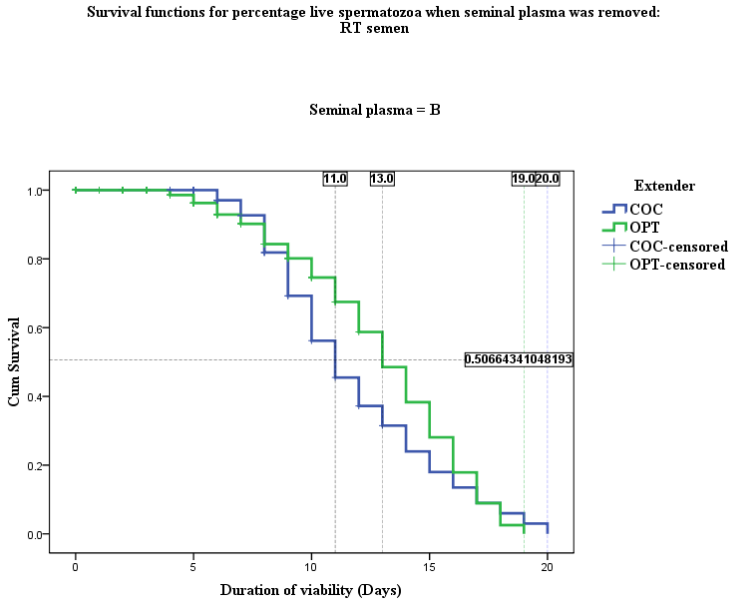
Figure 4.2 Kaplan Meier plots of Survival functions for RT buck semen in different extenders when seminal plasma was intact

(a) = plots for percentage motile, (b) = plots for percentage live spermatozoa

OPT recorded an apparently longer duration for motile (a) and live (b) spermatozoa when seminal plasma was intact for RT semen. However, there were alternations of survival distribution of spermatozoa in the extenders over the storage period, as indicated by the interceptions of the curves. Any observed differences in the curves were not significant ($p>0.05$).



(a)



(b)

Figure 4.3 Kaplan Meier plots of Survival functions for RT buck semen in different extenders in absence of seminal plasma

(a) = plots for percentage motile spermatozoa, (b) = plots for percentage live spermatozoa

The survival distribution of percentage motile spermatozoa was apparently higher and had longer survival time in semen extended in OPT and compared to semen extended in COC, when semen without seminal plasma was stored at room temperature, (Figure 4.3 (a) above). A similar scenario was observed with survival distribution and survival time for percentage live spermatozoa (b). However the observed differences in the curves had no statistical significance (Table 4.9). The results therefore implied that when seminal plasma was removed both extenders had the same capability of maintaining spermatozoa motile and live for a similar duration.

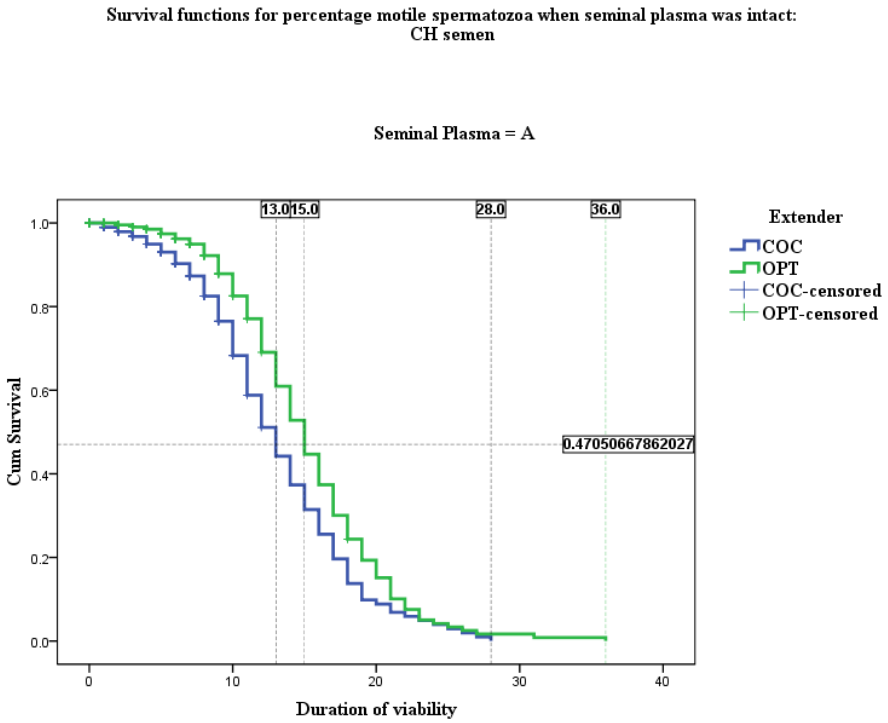
4.5.2 Analysis of effect of extenders on survival time of semen stored chilled

Table 4.10 Analysis of mean survival time and test of survival time for percentage motile spermatozoa in different extenders for CH buck semen

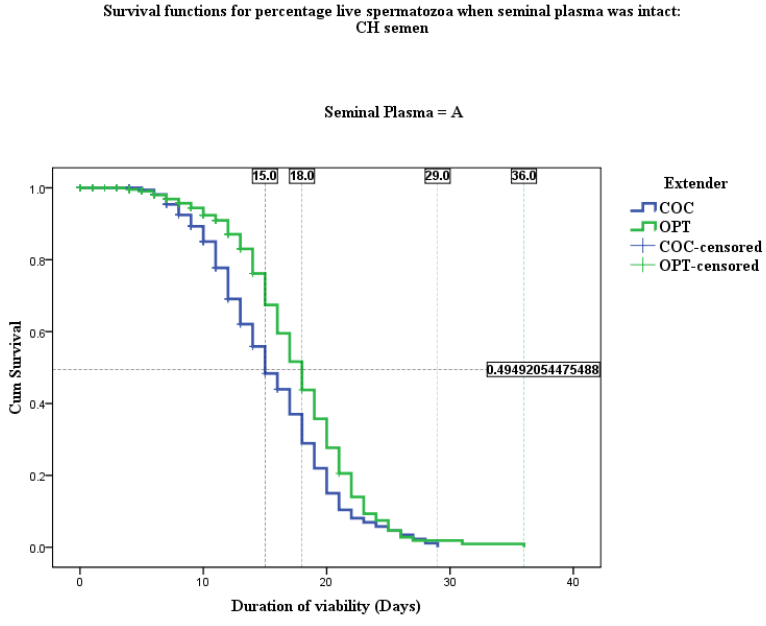
Viability parameter	**Seminal plasma**	**Extender**	**Percent censored**	**Mean[a]**		**Log Rank (Mantel-Cox)**			
				Estimate	Std. Error	COC		OPT	
						χ^2	Sig.	χ^2	Sig.
Motility	A	COC	87.6%	10.05	0.48			0.09	0.77
		OPT	88.3%	11.92	0.87	0.09	0.77		
Percentage	A	COC	94.8%	25.33	1.03			3.52	0.06
live		OPT	96.2%	29.69	1.72	3.52	0.06		
Motility	B	COC	90.9%	14.66	0.57			3.02	0.08
		OPT	92.8%	15.81	0.32	3.02	0.08		
Percentage	B	COC	96.9%	34.22	1.02			0.27	0.60
live		OPT	97.3%	34.0	0.65	0.27	0.60		

a. Estimation is limited to the largest survival time if it is censored.

From Table 4.10 above, the estimated mean survival time and distribution of both percentage motile and live spermatozoa were not statistically different between the two extenders irrespective of the seminal plasma status ($p>0.05$).



(a)

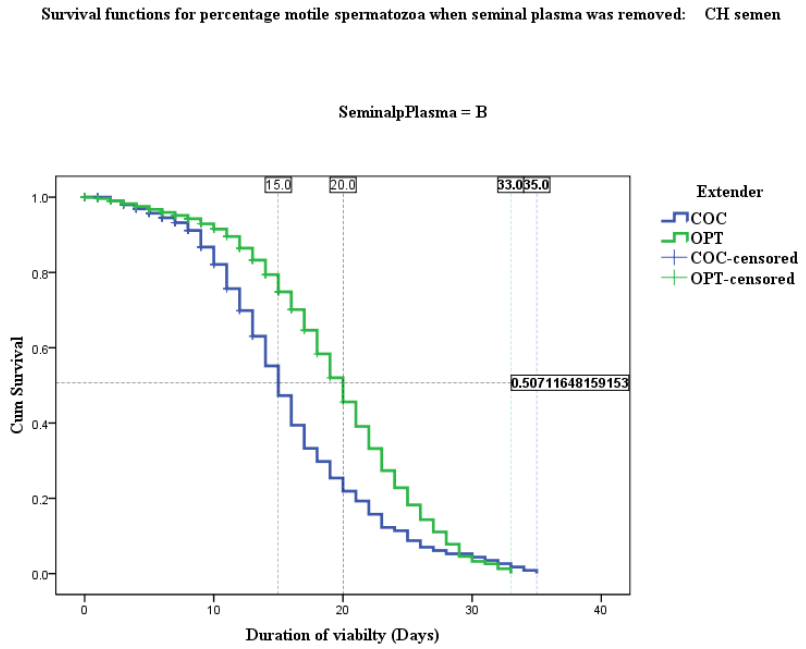


(b)

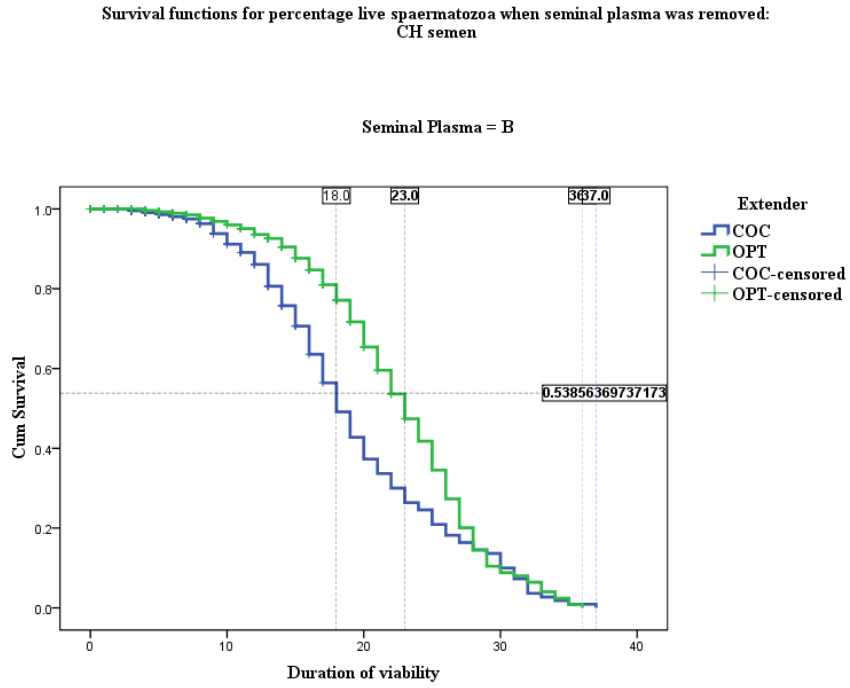
Figure 4.4 Kaplan Meier plots for Survival functions for CH buck semen in different extenders when seminal plasma was intact

(a) = plots for percentage motile spermatozoa, (b) = plots for percentage live spermatozoa

The survival distribution of percentage motile spermatozoa was apparently better in OPT than in COC when semen was stored chilled with intact seminal plasma as shown by the curves in Figure 4.4 (a) above, but the observed differences in the curves were not significant ($p>0.05$) (Table 4.10). When seminal plasma was left intact, OPT had an apparently better capability of maintaining spermatozoa live for longer duration for semen stored chilled as shown by the curves in (b) above the difference was not significant ($p>0.05$) (Table 4.10).



(a)



(b)

Figure 4.5 Kaplan Meier plots of Survival functions for CH buck semen in different extenders in absence of seminal plasma

(a) = plots for percentage motile, (b) = plots for percentage live spermatozoa

As illustrated in Figure 4.5 (a) and (b) above, semen extended in OPT apparently remained motile and live for a longer period when seminal plasma was removed and semen samples stored chilled. However, the differences in survival distributions for percentage motile and live spermatozoa were not significant (motility: p=0.08; percentage live p= 0.6) (Table 4.10).

4.5.3 Analysis of effect of seminal plasma on survival time and distribution of buck semen stored at room temperature

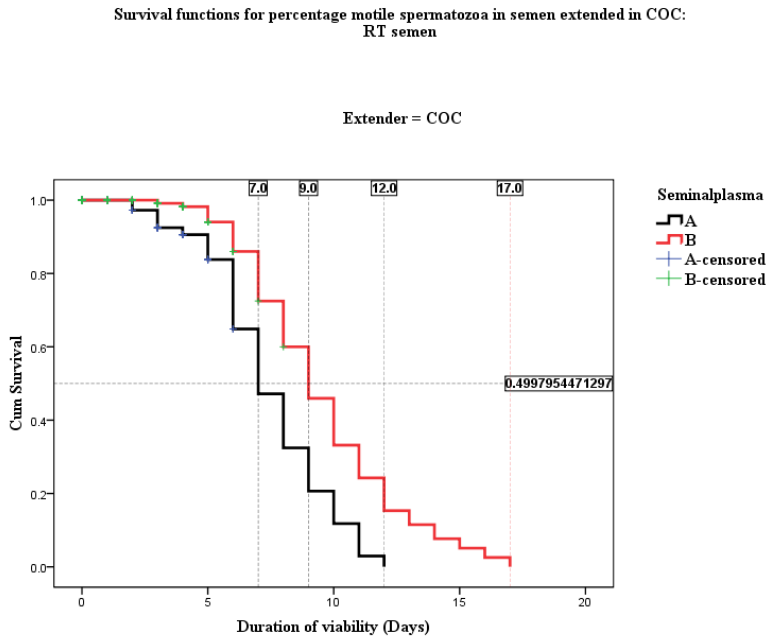
Analysis was done to establish the effect of seminal plasma on motility and percentage live spermatozoa for semen extended semen stored at room temperature (RT semen) and stored chilled (CH semen). The results were presented in tables and survival distribution plots below.

Table 4.11 Mean survival time and distribution for percentage motile and live spermatozoa for RT buck semen with and without seminal plasma

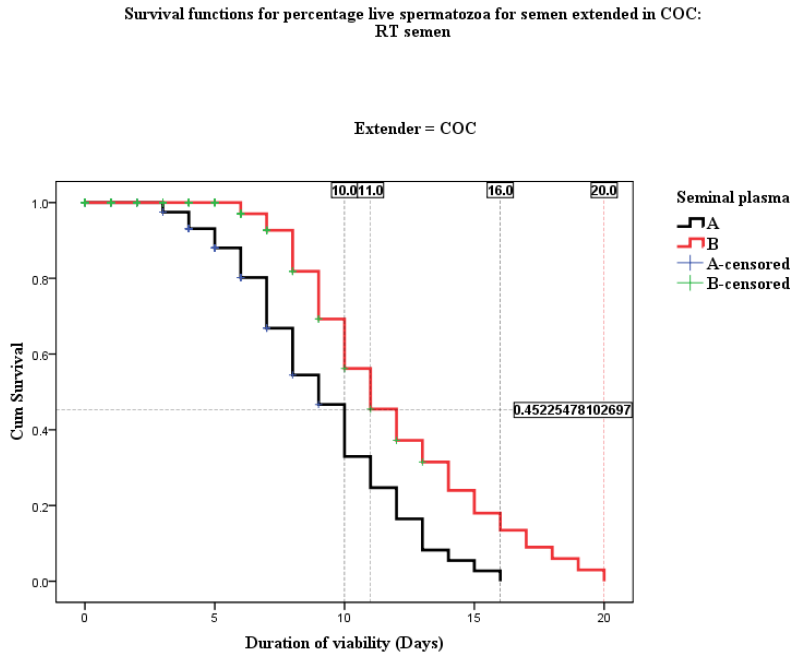
Viability parameter	**Extender**	**Seminal plasma**	**Percent censored**	**Mean[a]**		**Log Rank (Mantel-Cox)**			
				Estimate	Std. Error	A χ^2	Sig.	B χ^2	Sig.
Motility	COC	A	87.6%	10.1	0.48			19.52	0.0
		B	90.9%	14.7	0.57	19.5	0.0		
Percentage live	COC	A	90.4%	13.4	0.73			12.81	0.0
		B	93.6%	17.8	0.62	12.8	0.0		
Motility	OPT	A	88.3%	10.2	13.63			24.41	0.0
		B	92.8%	15.2	16.43	24.4	0.0		
Percentage live	OPT	A	90.2%	13.8	0.91			25.33	0.0
		B	94.1%	17.9	0.31	25.3	0.0		

a. Estimation is limited to the largest survival time if it is censored.

The estimated mean survival time (Mean±Sem) and distribution for motility and percentage live spermatozoa were significantly higher when seminal plasma was removed for RT semen ($p<0.05$) (Table 4.11 above)



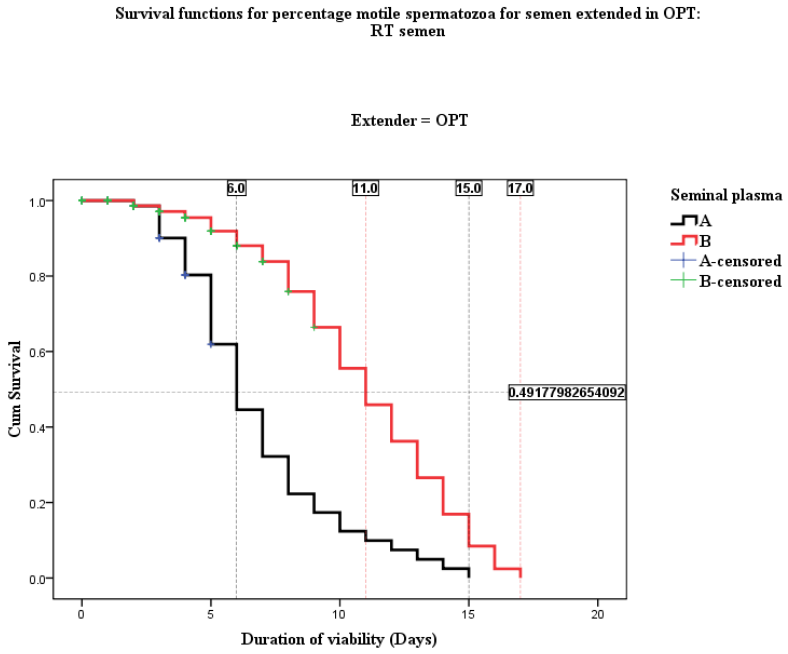
(a)



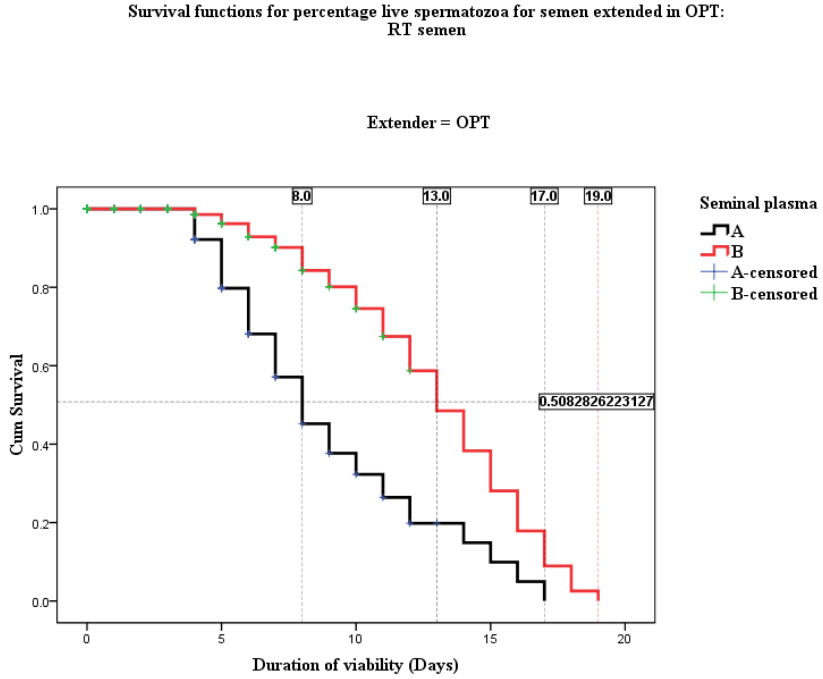
(b)

Figure 4.6 Kaplan Meier plots of Survival functions of viability parameters for RT buck semen extended in COC with (A) and without (B) seminal plasma.
(a) = plots for percentage motile spermatozoa, (b) = plots for percentage live spermatozoa

Removal of seminal plasma favoured the survival distribution of percentage motile and live spermatozoa for samples extended in COC and stored at room temperature as observed in Table 4.11. When seminal plasma was intact, COC maintained spermatozoa motile for shorter period (12 days) than when seminal plasma was removed (17 days) (Figure 4.6(a) above). When seminal plasma was intact, the duration of survival of live spermatozoa was shorter (16 days), compared to when seminal plasma was removed (20 days) (b).



(a)



(b)

Figure 4.7 Kaplan Meier plots of Survival functions of viability parameters for RT buck semen extended in OPT with (A) and without (B) seminal plasma

(a)= plots for percentage motile spermatozoa, (b) = plots for percentage live spermatozoa

Removal of seminal plasma improved the survival time of viability of semen extended in OPT and stored at room temperature ($p<0.05$)(Table 4.11).The survival time for motile spermatozoa was 15 days when seminal plasma was intact compared to 17days when seminal plasma was removed (Figure 4.7(a) above). At mean cumulative survival distribution of percentage motile spermatozoa, an estimated survival time of six days was recorded when seminal plasma was intact, compared to 11days when seminal plasma was removed. The survival time for percentage live spermatozoa was 17days when seminal plasma was intact and 19days when seminal plasma was removed (Figure 4.7 (b)).

4.5.4 Analysis of effect of seminal plasma on survival time and distribution of buck semen stored chilled

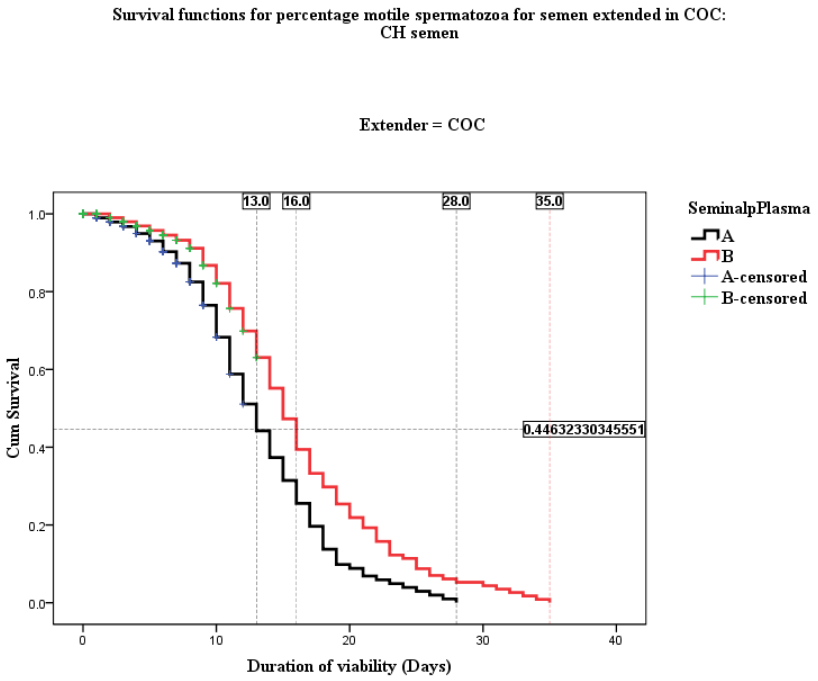
Analysis was done to compare the effect of seminal plasma on the survival distribution and estimated survival time of percentage motile and live spermatozoa for semen samples stored chilled. The results were as presented in Table 4.12 and Figures 4.8 and 4.9 below.

Table 4.12 Mean survival time and distribution for percentage motile and live spermatozoa for CH buck semen with and without seminal plasma

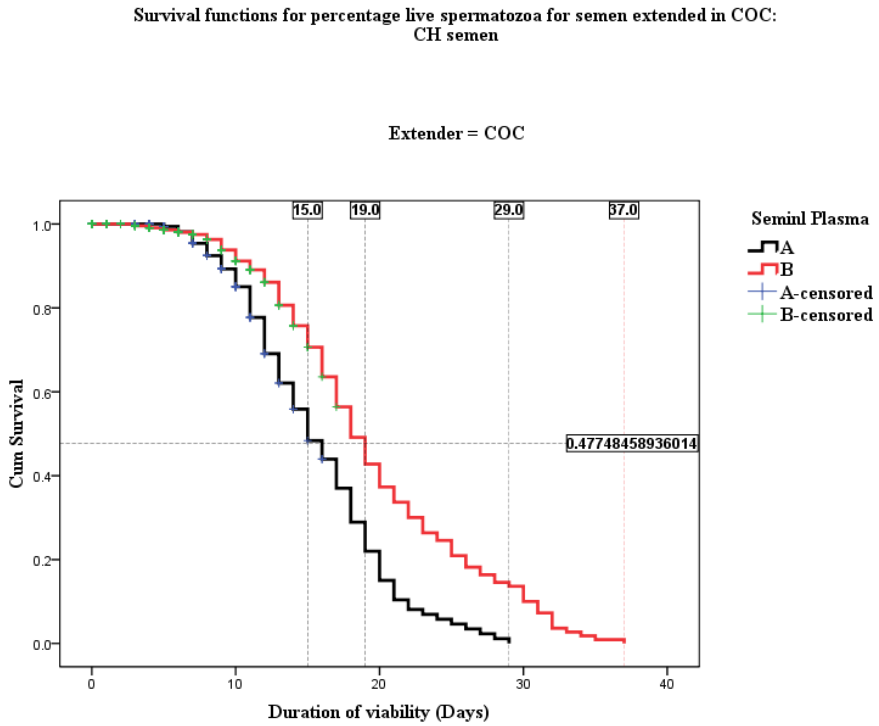
Viability parameter	**Extender**	**Seminal plasma**	**Percent Censored**	**Mean[a]**		**Log Rank (Mantel-Cox)**			
				Estimate	Std. Error	A		B	
						χ^2	Sig.	χ^2	Sig.
Motility	COC	A	94.2%	24.1	1.1			3.8	0.05
		B	95.2%	30.2	1.3	3.8	0.05		
Percentage live	COC	A	94.8%	25.3	1.0			14.1	0.0
		B	96.9%	34.2	1.0	14.1	0.0		
Motility	OPT	A	94.5%	27.8	2.1			13.6	0.0
		B	96.5%	31.1	0.6	13.6	0.0		
Percentage live	OPT	A	96.2%	29.7	1.7			12.6	0.0
		B	97.3%	34.0	0.7	12.6	0.0		

a. Estimation is limited to the largest survival time if it is censored.

As indicated in Table 4.12, removal of seminal plasma favoured the survival distribution and duration of percentage motile and live spermatozoa in each of the extenders when semen was stored chilled ($p<0.05$). This is indicated by significance of the differences in the survival distribution and duration in absence and presence of seminal plasma. COC had a weak difference in survival time and distribution of percentage motile spermatozoa when seminal plasma was removed and when intact ($p=0.05$), compared to OPT ($p=0.00$).



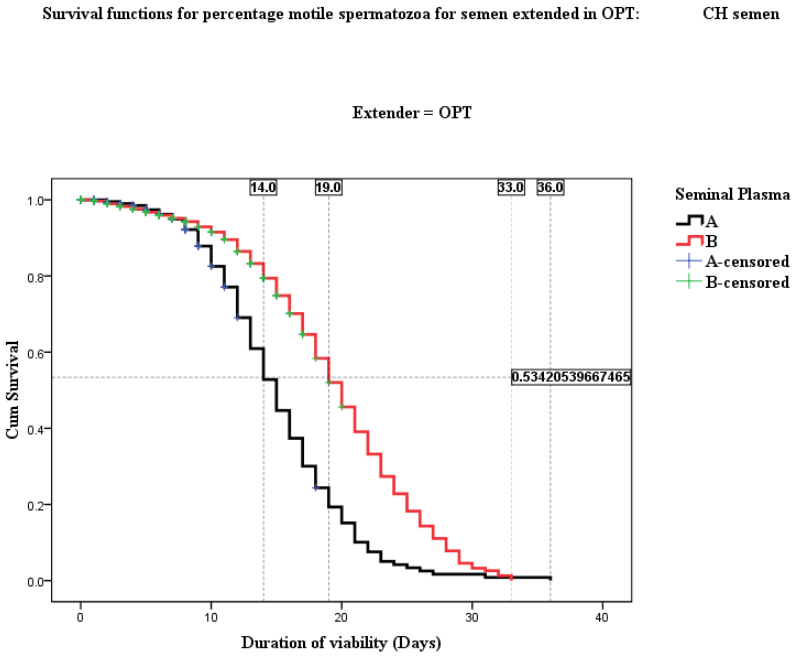
(a)



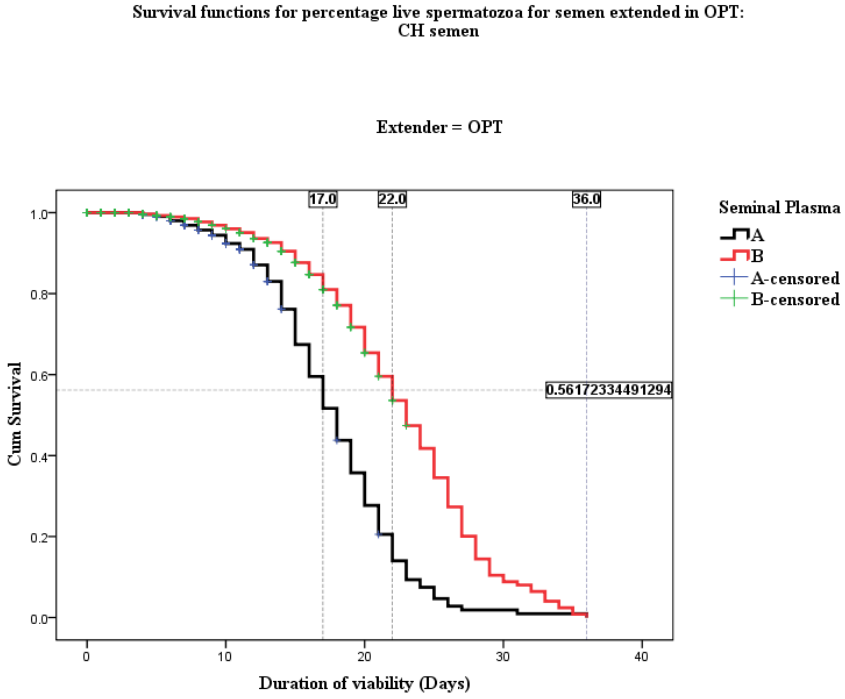
(b)

Figure 4.8 Kaplan Meier plots of Survival functions for viability parameters for CH buck semen extended in COC with (A) and without (B) seminal plasma
(a) = plots for percentage motile spermatozoa, (b) = plots for percentage live spermatozoa

When samples extended in COC were stored chilled, the survival distribution of percentage motile (Figure 4.8 (a)) and live (b) spermatozoawere better when seminal plasma was removed ($p<0.05$). Spermatozoa remained progressively motile for 35 and 28 days when seminal plasma was removed and when intact respectively (a). Similar ly they remained live for longer when seminal plasma was removed than when intact (29 and 37 days respectively).



(a)



(b)

Figure 4.9 Kaplan Meier plots for Survival functions for viability parameters for CH buck semen extended in OPT with (A) and without (B) seminal plasma

(a) = plots for percentage motile spermatozoa, (b) = plots for percentage live spermatozoa

Semen extended in OPT and stored chilled recorded motile cells for 33 days when seminal plasma was removed and 36 days when seminal plasma was intact (a) compared to 36 days recorded in each cases for percentage live spermatozoa (b).

4.6 Mean evaluation parameter values at 50% viability cut-off showing number of days the cut-off was attained

To conform to Kenyan standards at the Kenya Animal Genetic Resource Centre (K.A.G.R.C), a mean value of 50% (mean±SD) of viability parameters was considered in the current study. The number of days at which this cut-off was recorded under different ejaculate treatments was recorded as presented in Table 4.13 below.

Table 4.13 Mean values of evaluation parameter at 50% cut-off and the number of days semen samples took to reduce to the cut-off values

Parameter	**Temperature**	**Plasma**	**Extender**	**Days of Semen Storage**	**Mean±SD at 50% cut-off**	N
Motility	RT	A	COC	5	52.2±17.1	9
			OPT	3	54.0±21.7	10
		B	COC	5	54.0±11.7	10
			OPT	5	54.0±12.7	10
	CH	A	COC	6	50.0±9.7	10
			OPT	7	52.0±7.7	10
		B	COC	7	54±5.2	10
			OPT	10	51.0±8.8	10
%Live cells	RT	A	COC	5	62.34±15.7	9
			OPT	4	66.23±28.7	10
		B	COC	7	54.77±17.9	10
			OPT	7	57.21±20.4	10
	CH	A	COC	9	57.85±22.5	10
			OPT	11	57.61±13.8	10
		B	COC	10	56.86±20.5	10
			OPT	18	52.33±16.8	10

From Table 4.13 above, motility was maintained above the acceptable of 50% for five days when semen was extended in COC and stored at room temperature with or without removal of seminal plasma. OPT managed three days when seminal plasma was intact and five days when seminal plasma was removed at similar storage temperature.

When semen was stored chilled, COC maintained motility up to 50% for six days when seminal plasma was intact and seven days when seminal plasma was removed while OPT managed seven and ten days respectively.

At room temperature, COC maintained spermatozoa live up to 50% for five days when seminal plasma was intact compared to seven days when seminal plasma was removed while OPT managed four and seven days respectively. When semen was stored chilled the number of days at which percentage live spermatozoa reduced to 50% increased. Nine days was recorded for COC when seminal plasma was intact compared to ten days when seminal plasma was removed. Eleven and 18 days were recorded respectively for OPT.

4.6.1 Effects of treatments (seminal plasma and extender type) on motility at 50% cut-off for semen stored at room temperature

Effects of seminal plasma, extender type and interactivity of seminal plasma and type of extender on motility were tested for semen stored at room temperature at 50% motility cut-off. Results were recorded as shown in Table 4.14 and estimates of marginal means plotted as shown in Figure 4.10.

Table 4.14 Effects of seminal plasma and extender type on motility at 50% motility cut-off for RT semen

Dependent Variable:	Temperature	Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Motility	RT	Plasma	3749.1	1	3749.1	12.6	0.00
		Extender	894.8	1	894.8	3.0	0.08
		Plasma*Extender	1228.0	1	1228.0	4.1	0.04

*=interaction of the variables

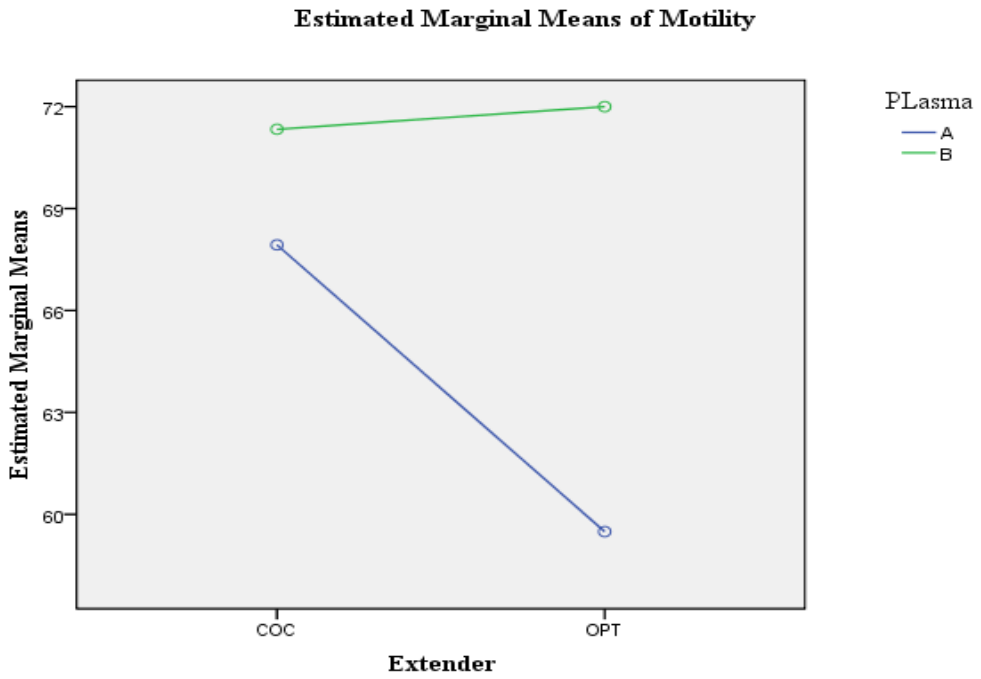


Figure 4.10 Marginal means of motility at 50% motility cut-off for buck semen stored at room temperature

(A) = semen extended seminal plasma, (B) = semen extender after removal of seminal plasma

At 50% motility cut-off, each extender recorded higher mean motility when seminal plasma was removed than when intact, for semen stored at room temperature ($p<0.05$). However, the difference in mean motility between the two extenders were not significant ($p=0.08$). Interactive effects of seminal plasma and extender type also affected significantly the observed changes in motility ($p<0.05$).

4.6.2 Effects of treatments (seminal plasma and extender type) on percentage live spermatozoa at 50% cut-off for semen stored at room temperature

Analysis of variance of values for percentage live spermatozoa was done up to 50% cut-off and results presented to show effects of treatment on semen viability parameter as shown in Table 4.15 below. Plots for estimated marginal means were also generated and is presented as in Figure 4.11.

Table 4.15 Effects of seminal plasma and extender type on percentage live spermatozoa at 50% cut-off for semen stored at room temperature

Dependent variable	Temperature	Source	Df	F	Sig.
Percentage live spermatozoa	RT	Plasma	1	7.4	0.007
		Extender	1	0.02	0.894
		Plasma *Extender	1	0.55	0.461

*=interaction of the variables

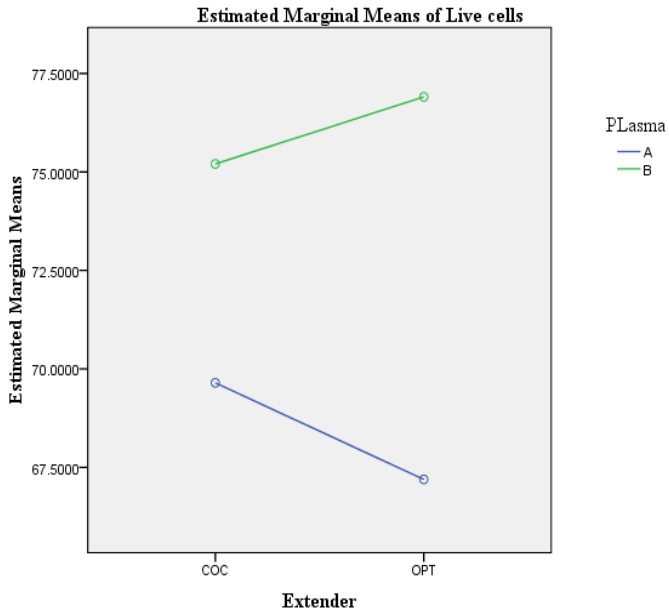


Figure 4.11 Marginal means of percentage live spermatozoa at 50% cut-off for buck semen stored at room temperature
(A) = semen extended seminal plasma, (B) = semen extender after removal of seminal plasma

At 50% motility cut-off, each extender recorded higher mean percentage live spermatozoa when seminal plasma was removed than when intact, for semen stored at room temperature ($p<0.05$). However, the difference in mean percentage live spermatozoa between the two extenders were not significant ($p>0.05$). Interactive effects of seminal plasma and extender type also did not affect mean percentage live spermatozoa ($p>0.05$).

4.6.3 Effects of seminal plasma and extender type on motility at 50% cut-off for buck semen stored chilled

Anova was run to establish the effects of seminal plasma and extender on motility at 50% motility cut-offs for semen stored chilled. The results presented in Table 4.16 showed the significance of the treatments on motility. A postulation of effects of such treatments to the population was presented as marginal mean plots (Figure 4.12).

Table 4.16 Effects of seminal plasma and extender type on percentage motile spermatozoa at 50% cut-off for buck semen stored chilled (CH)

Dependent variable	Temperature of storage	Source	Df	F	Sig.
Motility	CH	Plasma	1	19.1	0.000
		Extender	1	4.4	0.037
		Plasma *Extender	1	11.0	0.001

*=interaction of the variables

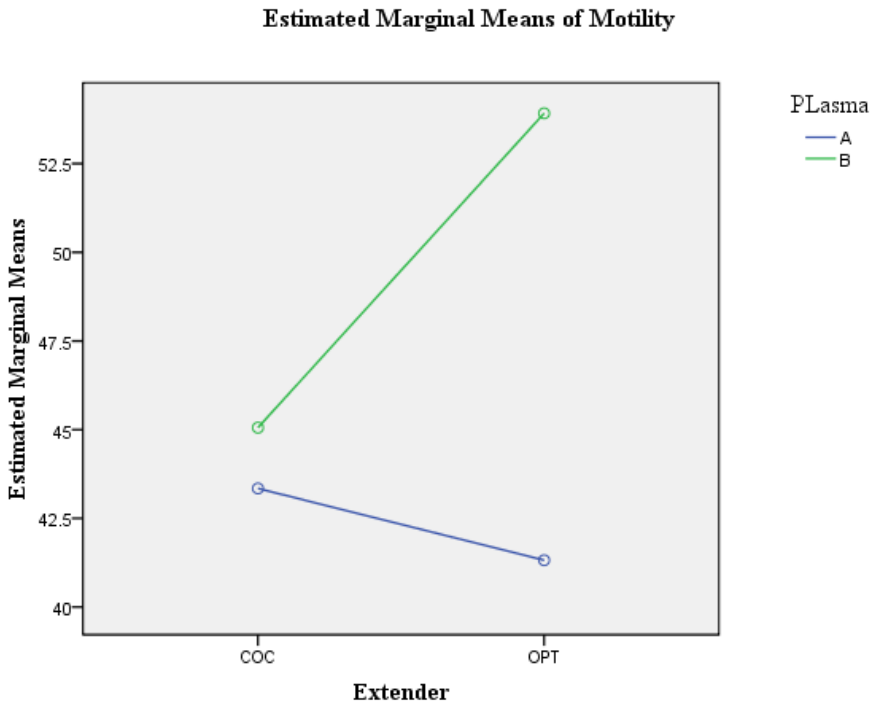


Figure 4.12 Marginal means of percentage motile spermatozoa at 50% cut-off for buck semen chilled

(A) = semen extended seminal plasma, (B) = semen extender after removal of seminal plasma

At 50% motility cut-off, each extender recorded higher mean percentage motile spermatozoa when seminal plasma was removed than when intact, for semen stored chilled ($p<0.05$) (Figure 4.12 and Table 4.16). OPT recorded higher mean motility values compared to COC ($p<0.05$). Interactive effects of seminal plasma and extender type also had significant effect on mean percentage motile spermatozoa at 50% cut-off ($p<0.05$) (Table 4.16).

4.6.4 Effects of seminal plasma and extender type on percentage live spermatozoa at 50% cut-off for buck semen stored chilled

ANOVA was run to establish the effects of treatments on percentage live spermatozoa at 50% cut-off for semen stored chilled. Results were presented in Table 4.17 and estimated marginal means plotted in Figures 4.13.

Table 4.17 Effects of seminal plasma and extender type on percentage live spermatozoa at 50% cut-off for buck semen stored chilled

Dependent variable	**Temperature of storage**	Source	**Df**	**F**	**Sig.**
Percentage live spermatozoa	CH	Plasma	1	19.8	.000
		Extender	1	17.8	.000
		Plasma *Extender	1	3.2	.072

*=interaction of the variables, source of the variable source

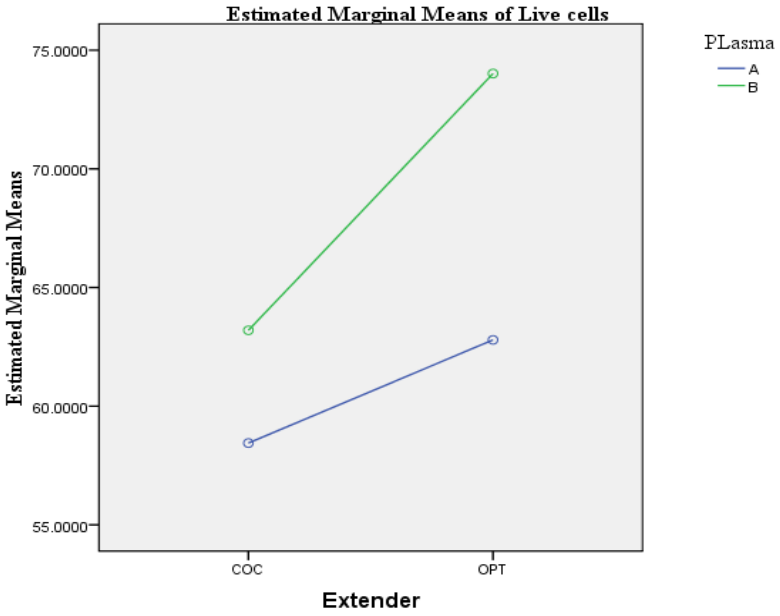


Figure 4.13 Marginal means of percentage live spermatozoa at 50% cut-off for buck semen chilled
(A) = semen extended seminal plasma, (B) = semen extender after removal of seminal plasma

At 50% motility cut-off, each extender recorded higher mean percentage live spermatozoa when seminal plasma was removed than when intact, for semen stored chilled ($p<0.05$) (Figure 4.13 and Table 4.17). OPT similarly recorded higher mean percentage live spermatozoa values compared to COC ($p<0.05$). However, interactive effects of seminal plasma and extender type had no effect on mean percentage live spermatozoa at 50% cut-off ($p>0.05$) (Table 4.17).

4.7 Evaluation of deep-frozen semen

Part of the semen samples were stored as deep-frozen semen (DF). Evaluation of these samples was done after 120 days of storage. Mean post-thaw motilities and percentage live spermatozoa are presented in Table 4.18. Effects of pre-storage treatments were presented in

Table 4.19. Plots of marginal means of evaluation parameters comparing bucks were as presented in Figures 4.14-4.17.

Table 4.18 Mean values of evaluation parameters for deep-frozen buck semen extended in COC and OPT with (A) and without (B) seminal plasma

Parameter		**Type of Extender**	**Seminal Plasma**	**Mean**	**Std. Deviation**	**N**
Deep-frozen Motility	Semen	COC	A	0.0	0.0	10
			B	0.0	0.0	10
		OPT	A	58.5	11.6	10
			B	57.0	7.2	10
Deep-frozen Percentage Live	Semen	COC	A	0.0	0.0	10
			B	0.0	0.0	10
		OPT	A	58.8	10.7	10
			B	56.4	8.2	10

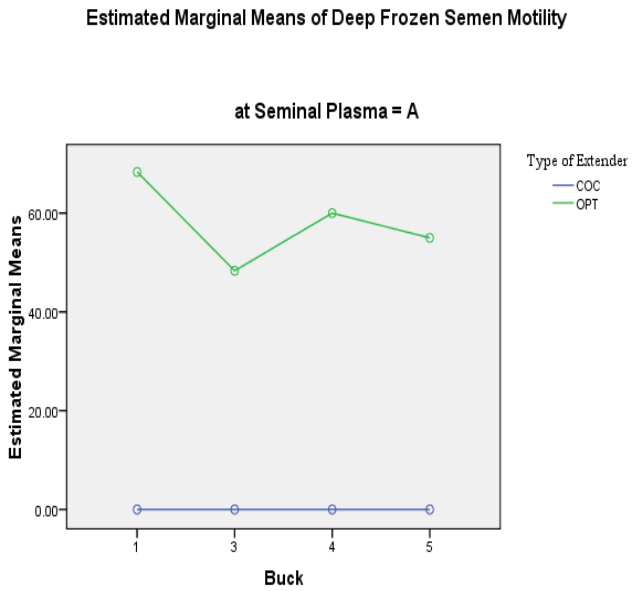
From Table 4.18 above, samples extended in OPT had a mean post-thaw motility of 58.5±11.56% when seminal plasma was intact (AOPT) compared to 57±7.15% when seminal plasma was removed (BOPT). The mean percentage live spermatozoa of 58.80±10.73% was recorded for semen samples extended in OPT with intact (AOPT) compared to 56.42±9.41% recorded when seminal plasma was removed (BOPT). COC did not support viability of the spermatozoa irrespective of removal or non-removal of seminal plasma in the current study.

Table 4.19 Effects of extender and seminal plasma on evaluation parameters (percentage motility and percentage live spermatozoa) for deep-frozen buck semen

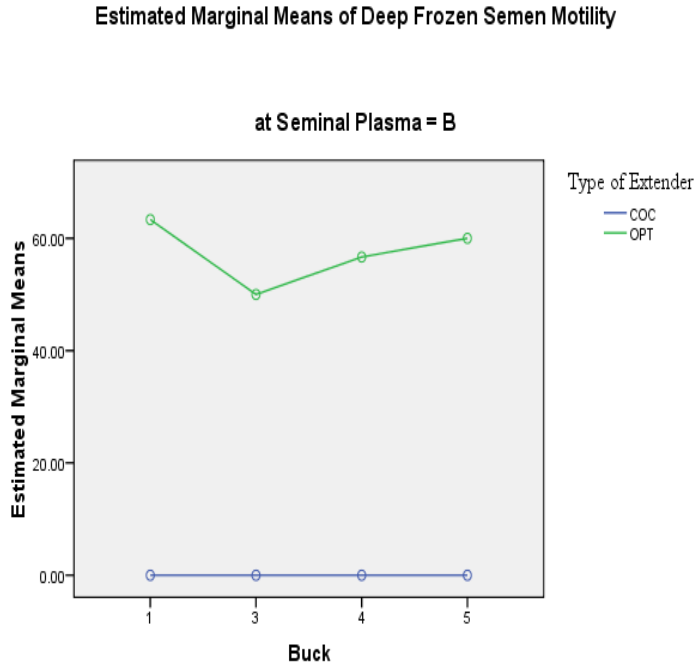
Source	Dependent Variable	Df	F	Sig.	Observed Power
Extender	Deep-frozen Semen Motility	1	722.2	0.0	1.000
	Deep-frozen Semen Percentage Live	1	722.9	0.0	1.000
Plasma	Deep-frozen Semen Motility	1	0.1	.73	.063
	Deep-frozen Semen Percentage Live	1	0.3	.58	.084
Extender * Plasma	Deep-frozen Semen Motility	1	0.1	.73	.063
	Deep-frozen Semen Percentage Live	1	0.3	.58	.084

*= interaction of the variables

From Table 4.19 above, extender type had significant effect on percentage motile and live spermatozoa for DF semen ($p<0.05$). Seminal plasma and the interaction of seminal plasma and extender type did not have any significant effects on the viability evaluation parameters ($p>0.05$).



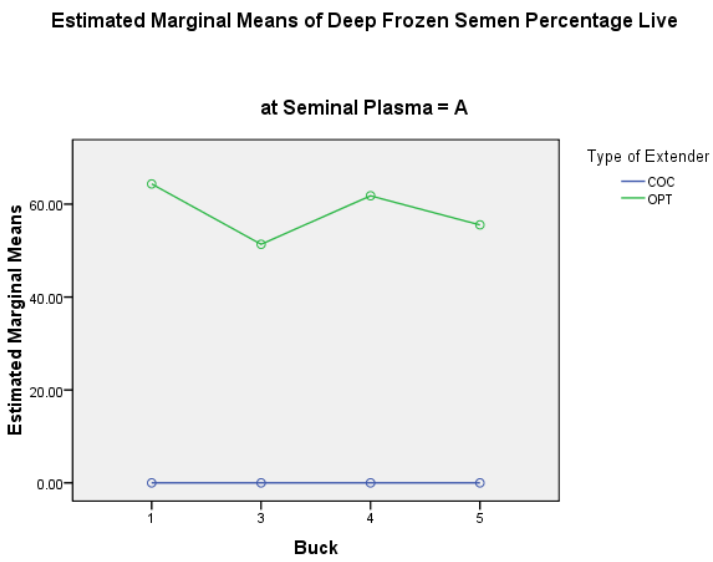
(a)



(b)

Figure 4.14 Marginal mean post-thaw motilities of semen with seminal plasma (a) and without seminal plasma (b), comparing bucks

From the above figures 4.14 a and b, Buck1 had the best marginal mean motility for semen extended in OPT in both cases of presence or absence of seminal plasma. This was followed by bucks 4, 5 and 3 respectively.



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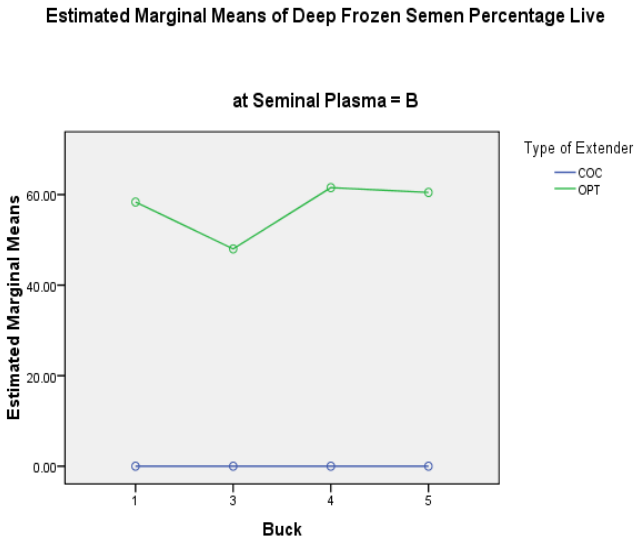

(b)

Figure 4.15 Marginal mean post-thaw percentage live spermatozoa with seminal plasma (a) and without seminal plasma (b), comparing bucks

As illustrated in Figures 4.15 a and b, when marginal means for post-thaw percentage live spermatozoa among bucks was compared, a similar trend observed with motility was recorded with buck1 leading followed by bucks 4, 5 and 3, irrespective of seminal plasma status.

CHAPTER 5.0: DISCUSSION

The current study investigated the effect of two extenders (COC and OPT) and seminal plasma on viability (motility; percentage live spermatozoa) of buck (goat) semen stored at room temperature (RT), chilled (CH) and deep-frozen (DF). Daily assessment of viability was done for RT and CH semen until zero values for viability evaluation parameters were recorded. Post-thaw viability of DF semen was assessed once after 120 days of storage.

The results from this study showed that the two extenders had equal capability of maintaining viability of buck semen both at room temperature and chilled whether seminal plasma was intact or removed. The results further indicated that removal of seminal plasma favoured the longevity of spermatozoa in each extender for both RT and CH semen. When semen was deep-frozen, COC did not support survival of goat semen regardless of presence or absence of seminal plasma. However, OPT maintained mean post-thaw motility and live spermatozoa above acceptable levels of 50%, in the presence and absence of seminal plasma.

For usability of such semen under the Kenyan semen production standards where the cut-off point for bull semen motility is 50%, RT goat semen extended in COC could be used within five days both when seminal plasma was removed and when seminal plasma was intact. Room temperature goat semen extended in OPT could be used within five days in absence of seminal plasma but within three days in presence of seminal plasma. Chilled goat semen extended in COC could be used within six days when seminal plasma was intact but for up to seven days when seminal plasma was removed. When extended in OPT chilled goat semen would be used for up to seven days in the presence of seminal plasma and for up to ten days in the absence of seminal plasma.

Semen extension is a critical step in any semen processing enterprise that focuses on mass production and storage. It is a common practice to evaluate fresh ejaculates for their suitability to undergo processing. Normally, ejaculates that do not meet acceptable standards are discarded. This study routinely examined such pre-treatment ejaculate parameters from the experimental bucks. The values of volume and individual motility (Sathe and Shipley 2014), concentration (Mushtaq *et al*., 2007), percentage live spermatozoa (Sule *et al.,* 2007), mass activity (Olayemi *et al*., 2011; Sathe and Shipley, 2014), and colour (Mushtaq *et al*., 2007), were within acceptable ranges. There was a significant buck effect on mass activity. This could be traced to relatively large fluctuation in mass activity values of buck 1 and 4 on different days of semen collection, which could have been due to individual difference in degree of response to similar sexual stimulation of the bucks (Mushtaq *et al*., 2007).

Collected ejaculates were extended using the two extenders and viability of semen analyzed within two hours with and without seminal plasma. Post-extension viability of semen is known to differ between extenders probably based on difference in their composition and species of animal from which semen is collected (Sathe and Shipley, 2014). The two extenders used in the current study maintained post-extension mean viability of spermatozoa above the acceptable levels of 50% with and without seminal plasma, as analyzed within two hours following extension without significant differences in mean motilities. This implied that semen with and without seminal plasma extended in each of the extenders could immediately be used for inseminations in the goat. There was however a significant reduction in motility within this time limit, a finding that was also reported by Sule *et al.,* (2007) in West African Dwarf goats. Immediate effect of extenders on sperm viability has been elaborately studied and is known to affect motility and live-dead ratio. Extenders interfere

with mitochondrial energy metabolism pathways, through poor control in production of Reactive Oxygen Species (ROS) among other factors (Kaya *et al.,* 2014). The effect is a reduction in motility vigour (Kaya *et al.,* 2014). Such known effects were therefore speculated to be responsible for similar results in the current study.

After confirming homogeneity of semen viability values within the GLM analytical system following extension, semen samples were stored. During storage, there was general progressive loss of viability of spermatozoa in RT and CH semen, over time. Survival function analysis of viability parameters showed normal decay of survival function curves for RT and CH semen. These results concurred with the observations by other workers for motility of buck semen stored at 4^0C and at room temperature (Peterson *et al*., 2007; Udeh and Oghenesode, 2011). Decrease in viability of semen with increased storage period has been associated with release of toxic reactive oxygen species (ROS) generated from damaged plasmalema, which decreases spermatozoa motility and modifies the morphology of the plasma membrane leading to capacitation and acrosomal reactivity (Kadirvel *et al*., 2009). These imply the sperm cell loses the biochemical composition of both the plasma membrane and the acrosomal membrane, compromising the functionality of the membranes. The decline in viability has further been associated with progressive depletion of nutrients such as potassium, sodium and proteins in the extender (Udeh and Oghenesode, 2011). In addition, metabolic activities of spermatozoa during storage have been demonstrated to result in a build up of toxic metabolic waste including phospholipid-free radicals that kill spermatozoa (Chatterjee and Gagnon, 2001; Agarwal *et al*., 2005; Munsi *et al*., 2007 and Wahjuningsih *et al*., 2012). It was therefore speculated that similar factors that detour survival of spermatozoa during storage could have also been responsible for the observed progressive decline in viability of spermatozoa in the current study.

In the current study, plasmalema integrity was tested through differential staining using eosin-nigrosin stain and live spermatozoa recorded as a percentage of the total number of spermatozoa counted under the microscope. The results showed a progressive loss of plasmalema integrity over the storage period as determines by the progressive decrease of percentage live spermatozoa. Compromised plasmalema integrity affects sperm cell metabolism which otherwise depends on energy supply in form of adenosine tri-phosphate (ATP) (Wahjuningsih *et al*., 2012). Plasma membrane regulates the transfer of substrate and electrolyte in and out of the cell during metabolic processes. Without intact plasma membrane, cells die out of defective energy metabolism (Bailey *et al.,* 2000). This further explained the trend of progressive reduction in the percentage live spermatozoa in the current study.

The current study observed longer duration of survival of semen stored chilled compared to that stored at room temperature. This was expected since at low temperatures there is usually slowed rate of cellular metabolic processes therefore nutrients are depleted at equally slower rate compared to room temperature. The resultant rate of toxic waste accumulation is therefore similarly low thus resulting in prolonged spermatozoa survival (Aboagla and Terada, 2003).

This study noted that during room temperature and chilled storage of extended semen, the two extenders, though different in their composition, showed similar longevity of spermatozoa both when seminal plasma was intact and when seminal plasma was removed. In the current study, COC was an egg-yolk containing coconut water-based extender while OPT was a soybean extender. Kumar (2016) documented OPTIXcell™ (OPT) as superior to egg-yolk-containing commercial extenders for preservation of bovine semen. The current study

however, compared viability of goat semen in the two extenders at the two different levels of temperature of storage and recorded equity.

The ability of any extender to support survival and motility of spermatozoa during storages is pegged on many factors that include its micronutrient composition and cryoprotective ability among others.

The positive performance of COC as constituted in the current study especially with room temperature and chilled semen was thought to be because of its components. One of its main components was coconut water. Coconut water-based extenders have been used for storage of semen from different animal species at lower temperatures. For instance, frozen boar semen (Luzardo *et al.,* 2010), refrigerated, and frozen semen of sheep, swine, bees and goat semen (Kotzias-Bandeira *et al*., 1999; Almeida and Soares, 2002; Gutierrez *et al*., 2006; Nejat *et al*., 2009; Daramola *et al.,* 2016a). Coconut water is known to contain sugars, amino acids, vitamins (C, B vitamins) and minerals (potassium, magnesium, zinc, selenium, iodine, sulfur and manganese) (Yong *et al*., 2009). Such components in any extender have been associated with favourable survival of spermatozoa. Sugars in coconut water which include glucose, sucrose and fructose (Saxelby, 2013); protect the plasma membrane from temperature related damage especially during low temperature storages. Such cryoprotective role, together with the ability of sugars to maintain osmotic balance and provide energy, normally support survival of spermatozoa during storage (Koshimoto and Mazur, 2002; Aboagla and Terada, 2003; Yancey, 2005; Naing *et al*., 2010). The amino acids contained in coconut water is known to protect the spermatozoa plasmalema from temperature related injury as well, through coating of the plasma membrane and combining with phospholipids on the plasma membrane thus improving the survivability of spermatozoa (Atessahin *et al*., 2008). Vitamin C (antioxidant) present in coconut water has been shown to reduce oxidative

stress to sperm cells taking advantage of its low toxicity and water solubility (Shen *et al.*, 2010; Daramola *et al.*, 2016a). Further, viability of spermatozoa in extenders containing coconut water has also been associated to its rich content of potassium (Mansour *et al*., 2002; Yong *et al.*, 2009). Potassium forms important components of cellular enzymatic reactions required for cellular metabolism thus favouring survival and speed of spermatozoa (Mansour *et al*., 2002). Though it was not within the scope of the current study to analyze the micronutrients composition of the coconut water used, it was speculated that these known advantages were partially responsible for the success of COC.

Egg yolk, which was one of the main components of COC in this study, is known to have cryoprotection ability because of its low-density lipoprotein (LDL) and lecithin content (Holt and Penfold, 2014). LDL stabilizes and forms a protective layer on the plasma membrane of spermatozoa. In addition, egg yolk incorporates its LDL, glycolipids and cholesterol in the plasma membrane thereby reducing the tendency of the membrane to change from liquid to gel phage during cooling (Holt and Penfold, 2014). These qualities were likely to have helped prolong the survival time of spermatozoa in COC especially in samples stored chilled. However, studies have demonstrated other characteristics of egg yolk that do not favour survival of spermatozoa. Egg yolk extenders are easily invaded and affected bacteria and xenobiotic contaminants in addition to such media containing some steroid hormones (Aires *et al.*, 2003). Egg yolk also contains High-Density Lipoproteins (HDL) and some minerals that inhibit cellular respiration thus affecting metabolic activities of spermatozoa leading to decreases in their motility and limiting their survival for a protracted period of time (Thun *et al.*, 2002). Since all the spermatozoa died eventually, then such negative effects could have been part of contributing factors.

The inclusion of antibiotics in COC was routinely done to protect samples from any invading bacteria during storage (Purdy, 2006). Therefore, the ability of COC to maintain viability of goat spermatozoa was associated with the advantages of its components.

OPT (OPTIXcell™) is a commercially available extender. It is a soybean lecithin based extender with no animal protein as indicated by the manufacturers. It contains soybean milk and lecithin therefore offers cryoprotective potential similar to egg yolk with comparatively lesser cryocapacitation and oxidative damage compared to egg yolk containing extenders (Singh *et al.*, 2012). This has been demonstrated in buffalo (Singh *et al.*, 2012), bull (Rehman *et al*., 2010), boar (Zhang *et al*., 2009), stallion (Papa *et al*., 2011) and ram (Forouzanfar *et al.*, 2010; Kasimanickam *et al*., 2011) semen. This study also found good results with OPT especially on both RT and CH buck semen storage.

In the current study, removal of seminal plasma significantly contributed to reduction of the percentage live spermatozoa. Various seminal plasma proteins have been implicated to lowered survivability of spermatozoa. Spermatozoa survive for long in the epididymis but once they get into contact with secretions of the accessory sex glands which compose the seminal plasma, they begin to deteriorate. Detrimental effect of seminal plasma has been associated with a 25-26 kDa seminal protein marker in the bull (Jobim *et al.*, 2004), a seminal plasma protein in alpaca (Marion *et al.*, 2010) and Ram Seminal Vesicular Protein – kDa 22 (RSVP22), in the ram (Manjunath *et al.*, 2009). In bovine (Therein *et al*., 1995), goat (Villemure *et al*., 2003) and bison (Biosvert *et al*., 2004) among other ungulates, these proteins which bind to spermatozoa during ejaculation and remain bound until sperm reaches oviduct, have been reported to be detrimental to survival of spermatozoa if they remain bound for a protracted period of time (Bergeron and Manjunath, 2006). They result in continuous removal of phospholipids and cholesterol from the surface membrane of spermatozoa

distorting the membrane structure thus compromising motility and eventually causing death of these cells (Bergeron and Manjunath, 2006). Removal of seminal plasma, thereby removing the detrimental proteins, has been found to enhance plasmalema integrity in both fresh and cooled spermatozoa from stallions (Barrier-Battut *et al*., 2013) and buffalos (Ahmad *et al.*, 1997) among other animal species. Similarly, the current study, speculated that the removal of such of detrimental proteins through the process of semen washing of the goat semen could have partly contributed to the better spermatozoa survival in each extender for RT and CH semen.

In addition to the known detrimental effects of seminal plasma protein to spermatozoa, there exists a known effect of egg yolk extenders specifically on goat spermatozoa in presence of seminal plasma (Leboeuf *et al.*, 2000; Sariözkan *et al*., 2010). In the current study, COC contained egg yolk. It supported semen viability better when seminal plasma was removed. It was therefore speculated that the poor performance of COC when seminal plasma was intact could have been contributed to by the unfavourable enzymatic interactivity.

The standard procedure of extending goat semen with extenders containing egg yolk has been removal of seminal plasma through centrifugation (Purdy, 2006). Other workers (Tuli and Holtz 1994; Cabrera *et al*., 2005; Peterson *et al*., 2007; Jiménez-Rabadán *et al*., 2012) however, have disputed the expected positive effect. Researchers have as well developed extenders devoid of any animal proteins such as OPTIXcell™. The current study tested the ability two extenders (COC- a formulated extender and OPT- a commercial extender) to maintain viability of buck spermatozoa with and without seminal plasma during storage. Removal of seminal plasma by centrifugation favoured survival of goat spermatozoa in semen samples stored at room temperature and chilled.

Further analysis was done to compare effects of seminal plasma, temperature of storage and extender type at ≥ 50% cut-off of viability values to concur with the practically acceptable Kenyan semen production standards. The means of evaluation parameters were recorded against the number of days taken for mean individual progressive motility to reduce to 50%. Progressive motility of spermatozoa is a critical assessment in breeding soundness of male animals. Spermatozoa have to be motile to be able to swim through the reproductive tract of the female and critical percentages of motile spermatozoa are considered for different animal species for successful inseminations. More than 30% progressively motile spermatozoa has been recommended for bulls, 60% for stallion and 70% for dogs for acceptance for such males in semen production centres (Ravafar and Moradi, 2013). The standard for the bull stud at the Kenya Animal Genetic Resource Centre (K.A.G.R.C) has been fixed at 50% for quality assurance purposes with bull semen. Based on duration of storage, usability of semen extenders have been classified as short (1-2 days), medium (3-4 days) and long term (5-6 days) extenders by Robert (**livestocktrail**.**illinois**.edu/uploads/.../papers/**Semen**%20Processing.pdf). From the results in the current study COC and OPT could generally be considered medium to long term extenders for preservation of RT buck semen. From the acceptable motility values, it would be logical to avoid the time consuming procedure of seminal plasma removal and use COC for extending buck semen for RT storage. For the Kenyan semen production industry where OPT is imported, it would even be more economical to use COC for goat semen extension since coconut fruits are readily and cheaply available.

Results on the usability of chilled goat semen indicated that removal of seminal plasma significantly increased the duration of motility. Ten days was recorded for motility in BOPT

versus seven days for AOPT, seven days was recorded for BCOC versus six days for ACOC. The number of days recorded were comparatively longer than those recorded for RT semen. The effect of seminal plasma protein on survivability was speculated even at low temperature of storage (Kozdrowski *et al*., 2007; Ustuner *et al*., 2009). The longer duration of viability could as well be associated with lowered rate accumulation of toxins in the extension media due to known low metabolic reactions at low temperatures.

In the Kenyan situation, these results were considered of extreme importance. For economic reasons, COC, if used with or without removal of seminal plasma, coupled with oestrus synchronization in goats, a great step forward would be achieved in expanding the use of artificial insemination in goats especially using chilled semen.

In the current study semen samples were also processed for storage as DF semen. Cyopreservation of semen in liquid nitrogen (-196^0C) is a common practice in many semen production set-ups. Cryopreserved semen maintains viability for many years (Malik *et al.*, 2015). However, this is dependent on the ability of components of semen extender to effectively protect spermatozoa against damages due to such low temperatures. In the current study, post-thaw mean percentage live and motile spermatozoa were compared across the extenders and seminal plasma status. Semen samples extended in COC were absolutely dead irrespective of seminal plasma status. There are similar reports of poor semen viability when coconut water is added to cryopreservation media for goat semen (Santillana and Garcia, 2000; and Gutierrez *et al*., 2006). On the contrary, Daramola *et al*., (2016a) reported favourable results for egg yolk-coconut (10% coconut water) extender. The success of incorporation of coconut water in extenders has been associated with its components including sugars, amino acids, vitamins and minerals known to have cryoprotective potential

in addition to providing energy and anti-oxidative ability (Mansour *et al*.,2002;Atessahin *et al*., 2008;Yong *et al*., 2009; Shen *et al.,* 2010; Daramola *et al.,* 2016a). Egg yolk as well, is known to have cryoprotection ability because it contains a low-density lipoprotein (LDL) and lecithin (Holt and Penfold, 2014). The current study tested the cryoprotective ability of coconut water and egg yolk without any addition of known penetrating cryoprotectants like glycerol and DMSO. However, the results showed that cryoprotective effect of coconut water and egg yolk, were not sufficient to maintain viability of deep-frozen semen.

It was concluded in this study that the commercially available extender (OPTIXcell™) was suitable for cryopreservation of goat semen. This was likely due to its cryoprotective components like phospholipids which are known to protect spermatozoa from water crystal formation within the cells (Stewart *et al*., 2015), among others which were not indicated by the manufacturer. Further studies need to be carried out on suitability of COC for deep-frozen semen.

CHAPTER 6.0: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

- This study showed that buck semen remained viable after semen was extended in each of the two extenders (COC and OPT).
- The newly formulated coconut based extender (COC) which was on trial for use in extending bull semen at K.A.G.R.C, had similar capability to maintain viability of goat semen as the commercial extender (OPT) at room temperature storage and semen stored chilled.
- Viability was maintained for significantly longer duration when extended semen was stored chilled than when stored at room temperature.
- Each extender significantly reduced the motility of spermatozoa in the first two hours of extension.
- Removal of seminal plasma favoured longevity of spermatozoa in each of the extenders when semen samples were stored at room temperature (21-23^{0}C).
- COC performed poorly when semen was stored deep-frozen.
- Semen extended in OPT and stored deep-frozen showed acceptable mean viability value (above 50%).

6.2 Recommendations

- Both extenders were recommended for extension of buck semen with or without removal of seminal plasma.
- It would also be beneficial to remove seminal plasma for better longevity of goat semen stored chilled and at room temperature.

- This study further recommends that RT semen be extended in any of the extenders without removal of seminal plasma and be used within five days for COC and three days for OPT instead of going through the rigorous process of seminal plasma removal that would give similar results especially for COC.
- OPT was recommended for DF semen and the tedious process of removal of seminal plasma be avoided.

6.3 Areas for further research

- Further study is needed to find out why the performance of the commercially available extender Optixcell^{TM} (OPT) was favoured by removal of seminal plasma in terms of its capability to maintain viability of spermatozoa.
- Conception rates should also be determined given the treatments on the semen samples.
- Further study is necessary to explore more on using coconut water based extender for deep freezing storage of goat semen through strengthening its ability to provide cryoprotection.

7.0: REFERENCES

Aboagla E.M. and **Terada T. (2003).** Trehlose-enhanced fluidity of the goat sperm membrane and its protection during freezing. Journal of Biological Reproduction, **69:** 1245-1250.

Agarwal A., Prabakaran S.A., and **Said T.M (2005)**. Prevention of oxidation stress injury to sperm. Journal of Andrology, **26**(6)**:** 654-660.

Ahmad M., Rehman S., Khan A., and **Ahmad K.M (1997)**. Effect of single and double washing on the liveability of buffalo bull spermatozoa at 37^0C. Pakistani Veterinary Journal, **17** (4):171-174.

Aires V. A., Hinsch K. D., Mueller-Schloesser R., Bogner K., and **Mueller-Scloedder S.,** and **Hinsch E.** (**2003**). *In vitro* and *in vivo* comparison of egg yolk-based and soybean lecitin-based extenders for cryopreservation of bovine semen. Journal of Theriogenology, **60**:269-279.

Aisen E., Medina V., and **Venturino A. (2002**). Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations. Journal of Theriogenology, **57**: 1801-1808.

Almeida R. and **Soares A. (2002)**. Usage of green coconut water and different tissue culture media for *in vitro* honey bee semen storage (*Apis mellifera* Hymenoptera: apoidea). Journal of Interciencia, **27:** 317-321.

Almin M., Druat X. and **Barrier-Batut I. (2010).** Osmotic resistance of frozen stallion spermatozoa in relation to fertility. Journal of Animal Reproduction Science, **121:** 130-132.

Amann R. (1999). Cryopreservation of sperm. In: Knobil, E., Neill, J.D. (Eds.), Encyclopedia of Reproduction. Academic Press, Burlington, MA.773–783.

Atessahin A., Bucak N., Tuncer B., and **Kizil M**. **(2008)**. Effects of antioxidant activities on microscopic and oxidative parameters of Angora goat semen following the freezing thawing process. Journal of Small Ruminants Research.**77**:38-44.

Austin W., Leidy R., Krise G., and **Hupp E. (1986).** Normal values for semen collected from Spanish goats by two methods. Journal of Applied Physiology, **24:** 823-827.

Azerˆedo, G., Esper, C., and **Resende, K.** (**2001**). Evaluation of plasma membrane integrity of frozen-thawed goat spermatozoa with or without seminal plasma. Journal of Small Ruminants Research, **41**: 257–263.

Bailey J.L., Jean-Francois B., and **Cormier N. (2000)**. Semen cryopreservation in domestic animals: A damaging and capacitating phenomenon. Journal of Andrology, **21** (1)**:1-7**.

Baracaldo M.I., Barth A.D and **Bertrand W. (2007).** Steps for Freezing Bovine Semen: From Semen Collection to the Liquid Nitrogen Tank. In: IVIS Reviews in Veterinary Medicine, I.V.I.S. (Ed.). International Veterinary Information Service, Ithaca NY, www.uesc.br/cursos/pos_graduacao/.../baracaldo_et_al_2007.pdf. Accessed on 23/11/2016.

Barrier-Battut, Bonnet C., Giraudo A., Dubois C., Caillaud M., and **Vidament M. (2013).**Removal of seminal plasma enhances membrane stability on fresh and cooled stallion spermatozoa. Journal of Reproduction in Domestic Animals, **48** (1): 64-71.

Bayemi P.H., Banla N. R., Leinyuy I., Niba A. T., Nsongka V. M., Mario G. and **Shamshuddin M. (2015).** Use of Fruits and Raffia Palm Sap (*Raffia hookeri*) in Chilled Bull Semen Extenders in Cameroon. Journal of Agricultural and Biological Sciences **1** (4): 142-149

Beltran G., Atabay P., Atabay C., Cruz M., Aquino P. and **Cruz C.** (**2013**). Optimized Extenders for Cryopreservation of Buck Semen for Artificial Insemination. Philippine Journal of Veterinary and Animal Sciences, **39** (1): 1-10.

Bergeron A. and **Manjunath P. (2006).** New insights towards the mechanism of sperm protection by egg yolk and milk. Journal of Molecular Reproduction Development, **73**: 1338-1344.

Biosvert M., Bergeron A., Lazure C., and **Manjunath P. (2004**). Isolation and characterization of gelatine-binding bison seminal vesicle secretory protein. Journal of Biological Reproduction, **70**: 656-661.

Blash S., Melican D., and **Gavin W. (2000**). Cryopreservation of epididymal sperm obtained at necropsy from goats. Journal of Theriogenology, **54**: 899-905.

Bispo C.A.S., Pugliesi G., Rodrigues M.T., Ker P.G., Filgueiras B., and **Carvalho G.R.** (**2011**). Effect of low and high egg yolk concentrations in the semen extender for goat semen cryopreservation. Abstract: Small Ruminant Research, **100**:54-58.

Brinsko S., Blanchard T., Spooner J., Love C., and **Varner D. (2011).** Relationships among morphologic characteristics and membrane integrity of stallion sperm. Journal of Clinical Theriogenology, **3:** 55-59.

Brito l., Barth A., Bilodeau-Goeseels S., Panich P. and **Kastelic J. (2003).** Comparison of methods to evaluate plasmalema of bovine sperm and their relationship with *in vitro* fertilization rate. Journal of Theriogenology, **60:** 1539-1551.

Burcu U., Ulgen G., and **Zekariya N.** (**2009**)**.** Effect of seminal plasma, egg yolk, and season on the freezability of saanen buck semen. Bulletin of the Veterinary Institute in Pulawy, **53**:369-374.

Cabrera F., **González F.**, **Batista M.**, **Calero P.**, **Medrano A.,** and **Gracia A. (2005).** The effect of removal of seminal plasma, egg yolk level and season on sperm freezability of canary buck (Capra hircus). Journal of Reproduction in Domestic Animals, **40** (3):191-195.

Cardoso R.C.S, Silva A.R., and **Silva L.D.M (2005).** Use of the powdered coconut water (ACP-106®) for cryopreservation of canine spermatozoa. Journal of Animal Reproduction **2**(4): 257-262.

Camus, A., Camugli, S., Leveque, C., Schmitt, E. and **Staub, C. (2011**). Is photometry an accurate and reliable method to assess boar semen concentration? Journal of Theriogenology, **75**:577-583.

Chatterjee S. and **Gagnon C. (2001).** Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Journal of Molecular Reproduction Development, **59**(4)**:** 451-458.

Corteel J. (1974). Viabilit´e des spermatozoids de bouc conserveset congel´es avec ou sans leur plasma seminal: effet du glucose (viability of goat spermatozoa deep-frozen with

or without seminalplasma: glucose effect). Ann. Biol. Anim. Biochem. Biophys., **14**: 741–745.

Courtens J., Nunes J. And **Corteel J.** (**1984**). Induction of the acrosome reactivity in the spermatozoa of the goat by secretions of the male accessory glands and milk. Journal of Molecular Reproduction Development. Gamete Research, **9** (3): 287–302.

Curry M. and **Watson P. (1994).**Osmotic effects on ram and human sperm membranes in relation to thawing injury. Journal of cryobiology, **31:** 39-46.

Daramola J.O., AdekunleE.O., Oke O.E., Onagbesan O.M., Oyewusi I.K., and **Oyewusi J.A. (2016a)**. Effects of coconut (*Cocos nucifera*) water with or without egg yolk on viability of cryopreserved buck spermatozoa. Journal of Animal Reproduction, Belo Horizonte, **13** (2):57-62.

Daramola J.O, Adekunle E.O, Iyasere O.S, Oke O.E., Sorongbe T.A, Iyanda O.A, Kehinde A.R, Aluko S.P, Olaoye I.O, Gbadebo O.E, Falolu L.I., Olukayode E.O., Ajayi R.A., Enikannaye O.J., and **Osunjaiye E.D**. **(2016b).** Effects of coconut milk alone or supplementation with pyridoxine in tris-extenders on viability of buck spermatozoa during vitrification. Journal of Small Ruminants Research, **136:**208-213.

DeJarnette J. and **Lefevre K. (2008).** Use of NucleoCounter in a semen processing laboratory. In: Proceedings of the 22nd technical Conference in Artificial Insemination and Reproduction. National Association of Animal Breeders, Columbia, Missouri. 89-93.

de la Cueva F., Pujol M., Rigau T., Bonet S.,Miro J., Briz M., and **Rodriguez-Gill J. (1997).** Resistance to osmotic stress of horse spermatozoa: the role of ionic

pumps and their relationships to cryopreservation success. Journal of Theriogenology, **48**: 947-968.

Donoghue A., Garner D., Donoghue D. and **Johnson L. (1996).** Assessment of membrane integrity of fresh and stored turkey spermatozoa using a combination of hypo-osmotic stress, fluorescent staining and flow cytometry. Journal of Theriogenology, **46:** 1543-1563.

Drobnis E., Nelson E. and **Burrill, M. (1980)**. Effect of several processing variables on motility and glutamic oxalacetic transaminase levels for frozen goat semen. I. Extender. Journal of Animal Science, **51**:439 (Abstract).

Emmens C. and **Blackshaw A. (1956).** Artificial insemination. Journal of Physiological Reviews, **36:** 277.

Evans G. and **Maxwell W. 1987**. Frozen storage of semen. In: Salamon's Artificial Insemination of Sheep and Goats. Butterworths, Wellington. 122–141.

Foote R.H. (2002). The history of artificial insemination: Selected notes and notables. Journal of American Society of Animal Science, 1-10.

Forouzanfar M., Hosseini S.M., Ostadhosseini S., Hajian M., Hosseini L., Abedi P., Nili N., Rahmani R.H., and **Nasresfahani M.H., (2010).** *In vitro* comparison of egg yolk-based and soybean lecithin- based extenders for cryopreservation of ram semen. Journal of Theriogenology, **73**: 480-487.

Fraser L. Gorszczaruk K. and **Streszezek J. (2001).** Relationship between motility and membrane integrity of boar spermatozoa in media varying in osmolality. Journal of Reproduction in Domestic Animals, **36:** 325-329.

Fukui Y., Kohno H., Togari T., Hiwasa M., and **Okabe, K.** (**2008**). Fertility after artificial insemination using a soya bean-based semen extender in sheep. Journal of Reproduction and Development, **54**:286-289.

Futino D., Mendes M., Matos W., Mondadori R., and **Lucci C., (2010).** Glycerol, methyl formamide and dimethyl-formamide in canine semen cryopreservation. Journal of Reproduction in Domestic Animals **45**, 214–220.

Gadea J. (**2003**). Review: Semen extenders used in the artificial insemination of swine. Spanish Journal of Agricultural Research, **1** (2):17-27.

GillerT., Buchwald P., Blum-Kaelin D., and **Hunziker W.**(**1992**). Two novel human pancreatic lipase proteins, hPLRP1 and hPLRP2. Difference in colipase dependence and in lipase activity. Journal of Biological Chemistry, **267**(21):6509–16516.

Gundogan M., Avdatek F., and **Yeni D.** (**2011**). Effect of Coconut milk, *Opuntia sp*. Milk and their combinations for cryopreservation of ovine semen. Journal of Arch. Zootec., **55:** 97-100.

Gutiérrez A., Palacios M., Jiménez C. and **Ramírez G., (2006)**. Coconut water *Opuntia* sp., milk and its combinations for the cryopreservation of ovine semen. *Archivos de Zootecnia*, **55**: 97-100.

Hackett A. and **Macpherson J. (2014)**.Some staining procedure for spermatozoa. In: Animal Andrology Theories and Applications. CAB International 2014.

Holt W. (2000). Basic aspects of frozen storage of semen. Journal of Animal Reproduction Science, **62**: 3–22.

Holt W. and **Penfold L. (2014)**. Fundamental and practical aspects of semen cryopreservation. In: Animal Andrology Theories and Applications. CAB International, 2014.

Iritani A. and **Nishikawa Y.** (**1961**). Studies on the egg-yolk coagulating factors in goat semen: II Properties of the coagulating factor and influential conditions for coagulation. In: Proceedings of Silver Jubilee Laboratory Animal Husbandry, Kyoto University, 97–104.

Iritani A. and **Nishikawa Y** (**1963**). Studies on the egg-coagulating enzyme in goat semen; on the position of yolk constituents attacked by the coagulating enzyme. Japanese Journal of Animal Reproduction, **8**:113–117.

Jiménez-Rabadán M., Ramóna O., García-Álvarezb A., Maroto-Moralesb E., del Olmob M., Pérez-Guzmána A., Bisbalb M., Fernández-Santosb, Gardeb J., and **A. Solerb** (**2012**)**.** Effect of semen collection method (artificial vagina vs. electroejaculation), extender and centrifugation on post-thaw sperm quality of Blanca-Celtibérica buck ejaculates. Journal of Animal Reproduction Science, **132**: 88– 95.

Jobim M.I.M., Oberst E.R., Salbego C.G., Souza D.O., Wald V.B., Tramontina F. and **Mattos R.C. (2004).**Two-dimensional polyacrylamide gel electrophoresis of bovine seminal plasma proteins and their relation with semen freezability. Journal of Theriogenology, **61**:255–266.

Kadirvel G., Kumar S., and **Kumaresan A. (2009).** Lipid peroxidation, mitochondrial membrane potential and DNA integrity of spermatozoa in relation to intercellular reactive oxygen species in liquid and frozen-thawed buffalo semen. Journal of Reproduction Science, **14** (1-3): 125-134.

Kasimanickam R., Kasimanickam V., Tibracy A., and **Pelzer K. (2011)**. Effect of semen extenders on sperm parameters of ram semen during liquid storage at 4^0C. Journal of Small Ruminants Research., **99**:208-213.

Kaya A., Sema B., Enwall L., and **Melili E. (2014).** Determinants of sperm morphology. Reactive Oxygen Species (ROS). In: Animal Andrology. Theories and Applications. CAB International, 34-56.

Koshimoto C. and **Mazur P. (2002).** The effect of the osmolality of sugar-containing média, the type of sugar, and the mass and molar concentration of sugar on the survival of frozen-thawed mouse sperm. Journal of Cryobiology, **45**:80-90.

Kotzias-Bandeira E., Waberski D., Weitze K. and **Barreto M. (1999).** Predilution and freezing of swine semen in natural coconut water at three different incubation treatments. Brazilian Journal of Veterinary Research in Animal Science **36:** 1-10.

Kozdrowski R., Dubiel A., Bielas W., and **Dzięcioł M. (2007).** Two protocols of cryopreservation of goat semen with the use of Computer-Assisted Semen Analysis System. Journal of Acta Veterinaria Brno., **76**: 601-604.

Kubovičová E., Ríha L., Makarevich A., Apolen D., Pivko J., Ríha L., Makarevich A., Apolen D.,and **Pivko J**. (**2010**). Effect of different semen extenders and additives to insemination doses on ewe´s pregnancy rate. Slovak Journal of Animal Science, **43**:2010 (3): 118 – 122.

Kulaksiz R., Ari1 U., Daşkin A., and **Üner A.** (**2013**). The effect of different glycerol concentrations on freezability of semen from Angora, Kilis and Saanen goats. Slovak Journal of Animal Science, **46** (2): 39-44.

Kumar A. (2016). Role of semen extender and motility enhancers in semen cryopreservation. www.slideshare.net/mobile.DrAnkeshkumar/. Accessed 15/1/2017**.**

Kumar K., Reddy B. and **Krishna K.** (**2013**). Comparison of different methods for assessing sperm concentration in infertility workup. International Journal of Medicine and Medical Sciences **5**(9): 396-400.

Kundu C., Chakraborty J., Dutta P., Bhattacharyya D., Ghosh A.and **Majumder G. (2000).** Development of a simple sperm cryopreservation model using a chemically defined medium and goat cauda epididymal spermatozoa. Journal of Cryobiology **40**: 117–125.

Kundu C., Das K., and **Majumder G. (2001)**. Effect of amino acids on goat cauda epididymal sperm cryopreservation using a chemically defined model system. Journal of Cryobiology, **41:**21–27.

Kundu C., Chakraborty J., Dutta P., Bhattacharyya D., Ghosh A. and **Majumder G. (2002).** Effect of dextrans on cryopreservation of goat cauda epididymal spermatozoa using a chemically defined medium. Journal of Reproduction, **123**: 907–913.

Leboeuf B., Manfred E., Boue, P., Piac`ere A., Brice G., Baril G.,Broqua C., Humblot P. and **Terqui M.**(**1998**). Artificial insemination of dairy goats in France. Journal of Livestock Production Science, **55**:193–202.

Leboeuf B., Restall B., and **Salamon S.** (**2000**). Production and storage of goat semen for artificial insemination. Journal of Animal Reproduction Science, **62**:113–141.

Liu C., Dong H., Ma D., Li Y., Han D., Luo M., Chang Z. and **Tan J. (2016).** Effects of pH during liquid storage of goat semen, on sperm viability and fertilizing potential. Journal of Animal Reproduction Science. (Abstract).

Lorton S. (1986). Determination of spermatozoa sperm concentration in initially extended bovine semen. In: proceedings, 11th Technical conference on Artificial Insemination

and Reproduction 77-79. National Association of Animal Breeders, Columbia, Missouri.

Lorton S. (2014). Evaluation of semen the Andrology laboratory. In: Animal Andrology Theories and Applications. CAB International. 101-135

Luzardo B.,Castro M.C., Gamboa F.A., Lopez M.A and **Lopez A.Y.** (**2010**). Osmolarity of coconut water (*Cocosnucifera*) based extenders and their effect over viability of frozen boar semen. American Journal of Animal and Veterinary Sciences, **5** (3): 187-191.

Malik A., Laily M. and **Zakir M.I. (2015).** Effects of long-term storage of semen in liquid nitrogen on the viability, motility, and abnormality of frozen thawed Friesian Holstein bull spermatozoa. Asian Pacific Journal of Reproduction **4**(1): 22-25.

Manjunath P., Lefebvre J., Jois P.S., Fan J., and **Wright M.W. (2009).** New nomenclature for mammalian BSP genes. Journal of Biological Reproduction, **80**: 394-397.

Mansour N., Lahnsteiner F.,and **Patzner A. (2002**).The spermatozoon of the African catfish: fine structure, motility, viability and its behaviour in seminal vesicle secretion. Journal of Fish Biology, **60**:545-560.

Marion I., Juyena N.S., Pasini G. and Stelletta C. (2010). Relazione tra le proteine del liquido seminale e la congelabilita` del seme nell'alpaca (Vicugna Pacos)—uno studio preliminare. Bologna, Italy: SIRA; 2010.

Memon M., Bretzlaff K. and **Ott R. (1985)**. Effect of washing on motility and acrosome morphology of frozen-thawed goat spermatozoa. American Journal Veterinary Research, **46**:473–475.

Menchaca, A; Pinczak, A. and **Queirolo, D. (2005)**. Storage of ram semen at 50C: effects of preservation period and timed artificial insemination on pregnancy rate in ewes. Journal of Animal Reproduction, **2**(3):195-198.

Morrel J.M., Johannisson A., Juntilla L., Rytty K., Backgren L., DalinA.-M., and **Rodriguez-Martinez H.** (**2010).** Stallion sperm viability, as measured by the nucleocounter SP-100, is affected by extender and enhanced by single layer centrifugation. Journal of Veterinary Medicine International, **10**: 1-7.

Munsi M., Buiyan M., and **Alam M. (2007).** Effects of exogenous glutathione on the quality of chilled bull semen. Journal of Reproduction in Domestic Animals, **42:**358-362.

Mushtaq A.M, Mickelsen W.D, and **Goyal H. O (2007**). Examination of the Reproductive tract and Evaluation of potential Breeding Soundness in the Buck. In: current Therapy in Large Animal Theriogenology 2nd ed. Sanders: Elsevier 515-518.

Naing W., Wahid H., Mohd Azam K., Rosnina Y.,Zuki B., Kazhal S., Bukar M., Thein M., Kyaw T., and **San M. (2010).** Effect of sugars on characteristics of Boer goat semen after cryopreservation. Journal of Animals Reproductive Science **122**:23-28.

Nejat N., Sijam K., Abdullah S., Vadamalia G., and **Dikinson M. (2009)**. Molecular characterization of a phytoplasma associated with Coconut Yellow Decline (CYD) in Malaysia. American Journal of Applied Science **6:** 1331-1340.

Nunes J., Corteel J., Combarnous Y. and **Baril G.(1982**). Rˆoledu plasma s´eminaldans la survie in vitro des spermatozo¨ıds de bouc. Reprod. Nutr.D´ev.**22**: 611–620.

Nynca J. and **Ciereszko A. (2009).** Measurement of concentration and viability of brook trout (*Salvelinus fontinalis*) spermatozoa using computer aided fluorescent microscopy. Journal of Aquaculture **292**: 256-258.

Olayemi F., Adeneji D. and **Oyeyemi M. (2011)**. Evaluation of sperm motility and viability in Honey-included Egg Yolk Based Extender. Journal of Global Science **7**(1):19-21.

Papa F.O., Felicio G.B., Melo-on C.M., Alvarenga M.A,.Vita B.D., Trinque C., Pouli-filho J.N.P. and **Dell'Aqua Jr. J.A. (2011).** Replacing egg yolk with soybean in the cryopreservation of stallion semen. Journal of Animal Reproduction Science **129**: 590-600.

Parish J. and **Foote R. (1987).**Quantification of bovine sperm separation by swim-up method: relationship to sperm motility, integrity of acrosome, sperm migration in polacrylamide gel and fertility. Journal of Andrology, **8:** 259-266**.**

Pellicer-Rubio M., Magallon T., and **Combarnous Y. (1997)**. Deterioration of goat sperm viability in milk extenders is due to a bulbourethral 60-kilodaltonglycoprotein with triglyceride lipase activity. Journal of Biology of Reproduction, **57**:1023–1031.

Pellicer-Rubio M. and **Combarnous Y. (1998).**Deterioration of goat spermatozoa in skimmed milk-based extenders as a result of oleicacid released by the bulbourethral lipase BUSgp60. Journal of Reproduction and Fertility, **112**: 95–105.

Peterson K., Kappen M., Ursem P., Nothling J., Colenbrander B. and **Gadella B.M.** (**2007**). Microscopic and flow cytometric semen assessment of Dutch AI- bucks: Effect of semen processing procedures and their correlation to fertility. Journal of Theriogenology, **67**:863-871.

Pinto C. and **Kozink D. (2008).** Simplified hypoosmotic welling test (HOST) of fresh and frozen-thawed canine spermatozoa. Journal of Animal Production Science, **104:** 450-455.

Purdy P.H. (**2006**). A review on goat sperm cryopreservation. Journal of Small Ruminant Research, **63**:215–225.

Quintero-Moreno A., Rubio J and **Gonzalez D. (2008).** Estimating cryodamage on plasma membrane integrity in bull spermatozoa using HOS-ENY test. Journal of Reproduction in Domestic Animals, **43 (5):** 62.

Rajamhendran R., Thangaraja M. and **Thangaraja P.** (**1981**). Comparison of three diluentents for preservation of buffalo semen at room temperature. Journal of Dairy Science, **23**:399–404.

Ravafar M.R. and **Moradi M.H. (2013)**

www.vivo.colostate.edu/hbooks/pathphys/reprod/semeneva/motility.html. Accessed on 17/1/2017.

Rehman FU., Zhao C., Shah M.A., Qureshi M.S. and **Wang X (2013).** Semen extenders and artificial insemination in ruminants. Journal of Veterinaria, **1** (1): 1-8.

Rehman F.U., Qureshi M.S., and **Khan R.U. (2010).** Effects of soybean on sperm parameters of Holstein-Friesian bull during liquid storage at 4^0C. Pakistan Journal of Zoology, **46** (1): 185-189.

Ritar A., Ball P., and **O'May P. (1990a)**. Artificial insemination of Cashmere goats: effects on fertility and fecundity of intravaginal treatment, method and time of insemination, semen freezing process, number of motile spermatozoa and age of females. Journal of Reproduction and Fertility Development, **2**: 377–384.

Ritar A., Ball P. and **O'May P. (1990b)**. Examination of methods for the deep freezing of goat semen. Journal of Reproduction and Fertility Development, **2**: 27–34.

Ritar A. and **Salamon S. (1982)**. Effects of seminal plasma and of its removal and of egg yolk in the extender on the survival of fresh and frozen-thawed spermatozoa of the Angora goat. Australian Journal of Biological Science, **35**: 305–312.

Robert K. Semen processing, extending & storage for artificial insemination in swine livestocktrail.illinois.edu/uploads/.../papers/Semen%20Processing.pdf. Accessed on 15/1/2017.

Roof D.J., Bowley S., Price L.L., and **Matsas D.J. (2012)**. Comparison of two commercial extenders for cryopreservation of goat semen without sperm washing. Abstract. Journal of Theriogenology, **77**(2):412-420.

Rota A., Bastianacci V., Maglli C., Panzani D. and **Camillo F. (2010)**. Evaluation of plasma membrane integrity of donkey spermatozoa. Journal of Reproduction in Domestic Animals, **45:** 228-233.

Salamon S. and **Ritar A. (1982)**. Deep freezing of Angora goat semen: effects of extender composition and method and rate of dilution on survival of spermatozoa. Australian Journal of Biological Science. **35**:295–303.

Santillana B. and **Garcia A. (2000).** Comparative study of two extenders for freezing Pelibuey sheep semen. Puebla. http://www.exopol.com/seoc/docs/vzq5536o.pdf. Accessed on 3/11/2016.

Saragusty J., Gacitua H., King R. and **Araw A.** (**2006**). Post-mortem semen cryopreservation and characterization in two different endangered Gazelle species (*Gazelle gazelle* and *Gazelle dorcis*) and one subspecies (*Gazelle gazelleacuiae*). Journal of Theriogenology, **60**:775-784.

Sariözkan S., Bucak M.N., Tuncer P.B., Tasdemir U., Kinet H., and **Ulutas P.A. (2010)**. Effects of different extenders and centrifugation/ washing on post-thaw microscopic-oxidative stress parameters and fertilizing ability of Angora buck sperm. Journal of Theriogenology, **73**: 316–323.

Sathe S. and **Shipley C.F. (2014)**. Applied Andrology in Sheep Goats and Selected Cervids. In: Animal Andrology Theories and Applications. CAB International, 226-253.

Sawyer D.E. and **Brown D.B.** (**1995**).The use on an in vitro sperm activation assay to detect chemically induced damage of human sperm nuclei. Journal of Reproduction and Toxicology, **9:** 351–357.

Saxelby C. (2013). Composition of coconut water. Foodwatch.com.au. Accessed on 1/4/2016.

Seré C., Ayantunde A., Duncan A., Freeman A., Herrero M., Tarawali S. and **Wright I. (2008)**. Livestock production and poverty alleviation – challenges and opportunities in arid and semi-arid tropical rangeland based systems. http://www.ilri.org/ILRIPubAware/Uploaded%20Files/Sere%20IGC%20paper%208%20pages%20final_formatted.pdf. Accessed on 14/8/14.

Shamsuddin M., Amiri Y. and **Bhuiyan M. (2000).** Characteristics of buck semen with regards to ejaculate numbers, collection intervals, extension and preservation periods. Journal of Reproduction in Domestic Animals, **35:** 53-57.

Shen J., Lai Q., Mattei J., Ordovas M., and **Tucker L**. (**2010**). Association of vitamin B6 status within inflammation, oxidative stress, and chronic inflammatory conditions: The Boston Puerto Rican Health Study. American Journal of Clinical Nutrition, **91**:337-342.

Singh A.K., Singh V.K., Narwader B.M., Mohanty T.K., and **Atreja S.K. (2012).** Comparative quality assessment of buffalo (*Bubalus bubalis*) semen chilled (5^0C) in egg yolk and soybean-based extender. Journal of Reproduction of Domestic Animals**,** **47:** 590-600.

Soe Win Naing, Abdi W. H., Mohd A.K.G., Rosnina Y., Zuki A.B., Kazhal S., Muhammad M.B., Myint T., Than K. and **Maung M.S.**(**2010**). Effect of seminal plasma removal, washing solutions, and centrifugation regimes on Boer goat semen cryopreservation. Pertanika Journal of Tropical Agricultural Science, **34 (**2**)**: 271 – 279.

Stewart J., Shipley C., Seder A., Canisso I., Po E., Ellerbrock R., and **Lima F. (2015).** Comparison of different extenders for cryopreservation of semen in white-tailed deer. 7th Annual Illinois Symposium on Reproductive Sciences. Abstract.

Sule W.F., Oyeyemi M.O, and **Akusu M.O.** (**2007**)**.**Coconut milk - citrate as extender for West African dwarf buck spermatozoa at room temperature. Journal of Biokemistri, **19**(2):65-73.

Therein I., Bleau G. and **Manjunath P. (1995)**. Phosphatidyl-choline binding proteins of bovine seminal plasma modulate capacitation of spermatozoa by heparine. Journal of Biological Reproduction, **52**: 1372-1379.

Thun R., Aurtado M. and **Janett F. (2002).** Comparison of BIOciphos-Plus® and Tris-egg yolk extenders for cryopreservation of bull semen. Journal of Theriogenology, **57:**1087-1094.

Tuli, R.K., and **Holtz, W.** (**1994**). Effect of glycerolization procedure and removal of seminal plasma on post-thaw survival and GOT-release from Boer goat spermatozoa. Journal of Theriogenology, **42**:547–555.

Udeh and **Oghenesode B.** (**2011**). Effects of Type of Extender and Storage Conditions on the Motility of Goat Spermatozoa. International Journal of Animal and Veterinary Advances, **3**(5): 282-286.

Upreti G.C., Hall E.L., Koppens D., Oliver J.E., and **Vishwanath R.**(**1999**). Studies on the measurement of phospholipase A2 (PLA2) and PLA2 inhibitor activities in ram semen. Journal of Animal Reproductive Science, **56:**107–121.

Ustuner B., Gunay U., and **Nur A. (2009)**. Effect of seminal plasma, egg yolk, and season on the freezability of Saanen buck semen. Bull Veterinary Institute Pulawy, **53**:369-374.

Vidal A.H, Batista A.M, da Silva E C, Gomes W.A, Pelinca M.A, Silva S.V, and **Guerra M.M. (2013).** Soybean lecithin-based extender as an alternative for goat sperm Cryopreservation. Journal of Small Ruminant Research, **109**: 47-51.

Villemure M., Lazure C. and **Manjunath P. (2003).** Isolation and characterization of gelatine-binding proteins from goat seminal plasma. Journal of Reproduction Biology and Endocrinology, **1**: 39-40.

Wahjuningsih S., Hermanto, Nuryadi, Budiarto A., and **Bhintoro P. (2012).** Effect of sperm concentration and length of storage at 5^0C on motility of goat spermatozoa. International Journal of Biological, Biomolecular Agriculture, Food and Biotechnological Engineering, **6** (6): 392-394.

www.imv-technologies.com. Accessed on 15/8/2014

Yancey H. **(2005)**. Organic osmolytes as compatible ,metabolic and counteracting cytoprotectants in high osmolarity and other stresses. Journal of Experimental Biology, **208**:2819-2830.

Yong H., Ge L., Ng Y., and **Tan N**. **(2009).** The chemical composition and biological properties of coconut (*Cocos nucifera* L.) water. Journal of Molecules, **14**:5144-5164.

Zhang S.S., Hu J.H., Li Q.W., Jiang Z.L., and **Xiao-Ying Z. (2009).** The cryoprotective effect of soybean lecithin on boar sperm quality. African Journal of Biotechnology, **8**: 6476-6480.

8.0: APPENDICES

Appendix 1: Sample of data collection table for semen parameters after collection

Date of collection	Buck ID	Semen volume	colour	Concentration	Mass Activity	Motility	Live cells	Dead cells

Appendix 2: Sample of data collection table for post-extension parameters of semen

Date of collection	Buck ID	Seminal plasma (A or B)	Extender (COC or OPT)	Motility	Live cells	Dead cells

Appendix 3: Sample of data collection table for daily semen sample assessments
Temperature of storage: RT/CH

Date of collection	Day of observation	Sample ID	Motility	Live cells	Dead cells

Appendix 4: Sample of data collection table for Deep-frozen semen

Date of collection	Sample ID	Motility	Live cells	Dead cells