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Effect of different equilibration periods pre-cryopreservation on post-thaw sperm motility Nguni and Boran bulls

by

Elizabeth van Staden

(BSc(Agric) Animal Science, University of Pretoria)

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Department of Animal and Wildlife Sciences

Faculty of Natural and Agricultural Sciences

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Supervisor: Prof. E.C. Webb

Co-Supervisor: Dr. M de la Rey

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This work is dedicated to my family.

Thank you for always believing in me and for encouraging me to follow my dreams, whatever it takes. Without your guidance, love and support, I would not have been able to achieve my goals.

To my parents, you are the best parents in the world, and I am honored to be called your daughter.

To my brother, thank you for always keeping me in touch with reality, you are the best little brother in the world.

To my other mom, I really miss you but I know that you are happy and smiling down on me where you are now.

All my love,

Lizelle

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SUMMARY

Compared to natural selection, the use of artificial insemination (AI) and other reproductive technologies rapidly increase the rate of genetic change in any population. In order to achieve success with AI, the semen used to inseminate cows must be of the highest possible quality. When semen is frozen, generally only about 50% of the spermatozoa survive the cryopreservation process. Thus, any factors possibly affecting the survival of spermatozoa through the numerous freezing-thawing steps should be studied, in order to identify the optimal conditions for the survival of spermatozoa.

The discovery of protective agents within egg yolk and glycerol was a major milestone in sperm cryopreservation. These agents protect bovine spermatozoa during cooling and freezing procedures and result in increased survival rates.

Cryopreservation of spermatozoa has become the most common technique for the preservation of male fertility of genetically superior sires even after their death. Using cryopreserved sperm to artificially inseminate females has become standard practice

in commercial dairy cattle herds and the application of this reproductive management tool is also expanding to beef herds worldwide. The use of glycerol as a cryoprotectant for bovine spermatozoa is credited as the reason for the success in bovine semen cryopreservation.

The purpose of this research was to quantify the effects of different cooling periods, as well as different glycerol equilibration periods on the post-thaw motility percentages and recovery fractions of semen collected from Boran and Nguni bulls. The research was subdivided into two experiments. In each experiment different cooling and glycerol equilibration times were researched. The first experiment involved shorter cooling times (30, 60, 120 and 240 minutes) with each cooling time followed by several longer equilibration times (4, 5, 6, 7 and 8 h). In the second experiment the cooling and equilibration times from the first experiment were reversed. This resulted in longer cooling times (4, 5, 6, 7 and 8 h) with each cooling time having shorter glycerol equilibration times (30, 60, 120 and 240 minutes). An egg yolk-Tris two-step extender was used in both the experiments.

The general trend for the glycerol equilibration periods studied in Experiment 1 was that the resulting overall average post-thaw motility percentage and average recovery fraction increased with longer periods. There was a breed difference when comparing the average post-thaw motility percentages after 4, 5, 6 and 8 h ($p<0.05$), while the average post-thaw motility percentages also tended to differ after 7 h of equilibration. The general trend observed for equilibration periods used in Experiment 2 was that the average post-thaw motility percentage increased as glycerol equilibration period increased up to 120 minutes, but after 240 minutes of glycerol equilibration, there was a slight decline. The differences in average post-thaw motility percentage after the respective glycerol equilibration periods were not statistically significant. The results of each experiment were used to create a matrix that can be used in practice. The matrix using results from Experiment 1 demonstrated that a cooling period glycerol equilibration period combination of 240 minutes and 7 h resulted in the highest (not

significantly different from most other combinations) average post-thaw motility rates. The matrix formed from the results of Experiment 2 demonstrated that an 8 h cooling period combined with a 60 minute glycerol equilibration period yielded the highest (not significantly different from most other combinations), average post-thaw motility percentage.

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AI Artificial Insemination

ASMA Computer Aided Sperm Head Morphology

AV Artificial Vagina

CASA Computer Assisted Semen Analysis

CFU Colony Forming Unit

CPA Cryoprotectant Agents

CSS Certified Semen Services

LDL Low Density Lipoprotein

NAAB National Association of Animal Breeders

ROS Reactive Oxygen Species

SCSA Sperm Chromatin Structure Assay

TOC Total Organic Carbon

Tris Tris hydroxymethyl aminomethane

CHAPTER 1

Research theme, hypothesis and motivation

Although there has been extensive research done on the subject of bovine semen cryopreservation, most of these studies were completed decades ago (Almquist, 1951; Dunn *et al.*, 1953; Graham *et al.*, 1956). Even today there are still discrepancies as to what the optimum equilibration periods have to be.

There has been significant progress in the components used for sperm extenders, and also the preparation of these extenders – which plays a role in determining the optimum equilibration periods for bovine semen cryopreservation (Almquist, 1954; Bean *et al.*,1963; Berndtson and Foote, 1976).

Another factor to consider is that the vast majority of the past research was done on European cattle breeds (*Bos Taurus*), and that the cryopreservation of semen from European and African breeds (*Bos Indicus*) may differ (Woods *et al.*,1986).

The aim of this study was to determine the effect of altering the standard equilibration periods recommended for the semen extender used during processing of semen from indigenous African cattle breeds. Several combinations of cooling and glycerol equilibration periods were evaluated and compared using post-thaw spermatozoa motility as the criteria.
The null hypothesis for both experiments was that there would be no significant differences in the post-thaw motility rates or recovery fractions of spermatozoa between different cooling and equilibration periods. The alternative for both experiments was that there would be significant differences in post-thaw motility percentages or recovery fractions of spermatozoa between the different cooling and equilibration periods.

The motivation for this study was thus to define and improve the freezing process of semen for cattle indigenous to Africa, in order to improve the post-thaw motility of spermatozoa and in turn the conception rates of cows inseminated with the

cryopreserved semen. This in turn may lead to farmers using artificial insemination in their herds, for accelerated genetic improvement of these indigenous cattle breeds.

CHAPTER 2
LITERATURE STUDY

2.1 Introduction

Throughout history man and animals have always competed for nutritional resources. Due to the demands of an ever-growing and evolving human population, livestock producers are constantly under pressure to supply more products, from even more limited resources. Thus, livestock producers are continuously searching for ways to improve the production efficiency of their livestock. This includes selecting for animals with better feed conversion ratios, disease resistance and improved reproductive efficiency. These factors can all be improved by genetic selection, in other words selecting and breeding only superior animals. This is known as genetic improvement (Bailey *et al.,* 2000; Salisbury *et al.*, 1978; Vishwanath, 2003). For genetic improvement to take place in any population there has to be an increase in both the gene frequency and gene combinations of the desired genes. This is achieved by selecting individuals possessing the desired traits and then manipulating their breeding. Reproduction rate is an important limiting factor in the rate of genetic progress, if the rate of reproduction can be improved, genetic intervals can be decreased, and thus genetic progress can also be accelerated. Compared to the cow, the potential selection intensity of a bull is much greater because of its reproductive capacity. A fertile and healthy bull can generally produce enough semen to breed more than 50 000 cows per year, when using artificial insemination (AI) (Salisbury *et al.*, 1978).

2.2 Artificial insemination (AI)

By performing AI, all of the above-mentioned factors can be improved. Particularly in the dairy industry, there has been a long history of using AI in progeny test systems. This has allowed for earlier identification of superior bulls and the rapid distribution of their genes into commercial herds worldwide (Vishwanath, 2003). A single ejaculate can be used to inseminate – and potentially impregnate – numerous females, thus maximizing the distribution of superior and desirable genetics. AI also eliminates the need for physical contact between animals,

therefore minimizing the distribution of sexually transmitted diseases (Bailey et al., 2000). Compared to natural selection, the use of AI and other reproductive technologies rapidly increases the rate of genetic change in any population. When used responsibly, these biotechnologies can result in great improvements of the livestock population, but there can also be disastrous consequences if applied without careful consideration of the possible impact to both the population and environment (Foote, 1999).

Foote (1998) summarised the potential genetic contribution of an individual sire using 2 equations:

Contribution of individual sire to genetic improvement =

No of progeny per sire X Genetic superiority of sire

Number of progeny per sire =

(No of sperm per sire / No of sperm inseminated per cow)

X % of potential semen used

X % of cows calving to a single insemination

Although AI has actually been practised in plant production for millennia – with the aid of flying insects and birds as inseminators – the ‘discovery’ of AI in animals was a much more recent event. AI is classified as the first great biotechnology technique to be applied in animal breeding, and it has had an enormous impact on the breeding practises in many species. It has also led to the development of many other reproductive technologies and ART’s.
Better management practises for both male and female animals, as well as improved procedures of collection, evaluation and preservation of semen and more accurate inseminating techniques are all developments that have contributed to the continuous success of AI (Foote, 2002).

In 1980 estimations were that the total amount of inseminations practised worldwide was upwards of 130 million. The application of AI worldwide is however still a growing practise, as demonstrated by a 1995 survey, that stated the total

inseminations worldwide for that year to be more than 200 million. In more than 95% of these inseminations frozen semen was used. (Vishwanath, 2003)

Using cryopreserved sperm to artificially inseminate females has become standard practice in commercial dairy cattle herds and the application of this reproductive management tool is also expanding in beef herds worldwide. The use of glycerol as a cryoprotectant for bovine sperm cryopreservation is credited as the reason for the success in bovine semen cryopreservation. This success is confirmed with the lower required insemination dose to still yield acceptable non-return rates for bovine, compared to other species (Medeiros *et al.,* 2002).

Cryopreservation of sperm has become the standard technique in preserving male fertility of genetically superior sires even after their death. In livestock, semen is collected from sires possessing a series of desirable characteristics, and females are then artificially inseminated with this collected semen to produce offspring with desirable traits. Generally a superior bull is able to produce up to 60 000 doses of semen per year, and by collecting and freezing this amount of semen, these sires can be exploited to achieve rapid genetic improvements in herds, worldwide (Curry, 2000; Ehmcke & Schlatt, 2008; Saacke, 1983).

There are significant differences between species in terms of the relationship between semen traits and fertility. Investigations into these differences commonly focus on sperm of the relevant species, specifically how to preserve the viability of the sperm cells. There are however, also differences between the females of different species – most importantly differences with regard to major obstacles for sperm on the way to the site of fertilization. The different sites of these barriers may lead to different sites of semen deposition when inseminating females. So for example, in cows the cervix serves as a selective screen for viable sperm, so the preferred site of semen deposition in cows will usually be past this point. The above-mentioned differences should be considered when evaluating the success of using cryopreserved sperm of different species (Saacke, 1983).

Another major development attributing to the success of AI in bovine was the method of recto-vaginal fixation of the cervix by Danish veterinarians. This allowed for the site of semen deposition to be deep within the cervix or even into the

uterine body. These researchers also found that by depositing semen closer to the potential site of fertilization and behind the major barrier, which is the cervix, fewer sperm are needed per insemination (Foote, 2002).

2.2.1 History of AI and semen cryopreservation

2.2.1.1 Early discoveries relating to AI

In 1677 Leeuwenhoek used a simple microscope to discover small motile cells which he named “animalcules”. This discovery was the cornerstone of any further research or discoveries that led to the development of current assisted reproductive technologies. The next notable event was the first documented case of AI which occurred more than 100 years later in 1784, when Spallanzani successfully inseminated a bitch. Spallanzani later also found that when semen is filtered, fertilization only occurred when the residue (sperm) left on the filter was used and not when the filtrate (seminal fluids etc.) was used. He also observed that when equine semen was cooled the sperm weren’t killed, but only remained motionless until the temperature was increased. Another century passed before the use of AI was also reported by Heape (1897) and several other researchers in independent studies on horses, dogs and rabbits. Heape was also credited with establishing the basis for the relationship between reproduction and seasonality (Foote, 1999; 2002).

2.2.1.2 Later discoveries related to AI and its possible uses

Ivanoff in 1899 was credited with initial attempts to establish AI as a breeding practise. By 1907 he had applied AI in the breeding management programmes for rabbits, dogs, foxes, poultry and domestic farm animals. As part of his research he trained technicians as inseminators and developed semen extenders to prolong the life of sperm. Ivanoff’s work also stimulated interest in AI outside Russia. In 1912 Dr. Ishikawa – a scientist who studied with Ivanoff – started an AI programme in Japan with horses. Later similar programmes for poultry, swine, goats, sheep and cattle, followed.

Milovanov later succeeded Ivanoff and established projects for the breeding of cattle and sheep in Russia. Milovanov also designed and made practical versions of the artificial vagina, specifically for these species as well as other practical

equipment for semen collection. Many apparatus currently used are still based on these designs (Foote, 2002).

The artificial vagina used in AI today was first devised by Amantea in 1914. It was originally meant for the collection of dog semen, but was quickly adapted for use in bulls, stallions and rams. Gunn experimented with using the electro-ejaculator to collect semen from male animals. This is an alternative method used when animals are not suited for semen collection using an artificial vagina. (Foote, 1999; Salisbury *et al.*, 1978)

Although the discovery of AI had occurred much earlier, it was not until the mid-1930's that all the necessary ingredients for the successful commercial applications thereof, were available. These 'ingredients' include an economic need to improve milk yield in dairy cows, as well as a measurement system for assessing the phenotypic expression of milk-producing capacity of cows and several other factors (Salisbury *et al.*, 1978). During the period of the 1940's and 1950's, there were various revolutionary discoveries regarding the semen cryopreservation process, as well as the storage environment for cryopreserved semen. This, together with new methods of sire selection, resulted in the application of AI to herds in remote regions that did not have access to viable collected semen previously (Vishwanath, 2003). When the first calf emanating from AI with cryopreserved semen was born in 1951, the technique was already starting to be incorporated into commercial breeding management programmes, and it established itself rapidly thereafter (Curry, 2000). With time, the process of AI became more accessible, and by the next decade, the majority of replacement stock was the result of applying AI (Vishwanath, 2003).

Several discoveries and developments played a crucial role in establishing the basic principles for the freezing of bovine semen. Phillips developed a phosphate-buffered egg yolk based extender in 1940, and a year later Salisbury *et al.* improved on this by using a citrate buffer that resulted in a clearer medium. This allowed for more accurate and critical sperm evaluation. A few years later, in 1948, two independent reports by Foote and Almquist described the remarkable results achieved when adding antibiotics to the semen extender. When Polge *et al.* discovered the cryoprotective properties of glycerol in 1949; it was the beginning

of a new era. Semen could now be frozen and stored for long periods, while still yielding acceptable fertility results (Foote, 1999). The discovery of protective agents within egg yolk and glycerol was a major milestone in sperm cryopreservation. These agents protected bovine sperm during cooling and freezing procedures and resulted in increased survival rates (Medeiros *et al.,* 2002).

Over the last few decades research has concentrated on the cryopreservation of bovine sperm, but this did very little to advance the preservation of any other body cells. This is partly due to the slow realization that bovine sperm cells are unique in its composition, and a lack of methods to measure damage caused to sub-cellular compartments (Hammerstedt *et al.* 1990).

2.2.1.3 New and potential trends in the livestock AI industry

There is a general negative relationship between fertility and production traits in cattle. The result of sustained selection for improved production traits in the dairy industry resulted in a decline in the reproductive efficiency of dairy herds using AI. By developing semen cryopreservation techniques that limit the rate of capacitation or prolong the post-thaw survival time of cryopreserved sperm, the sensitivity of insemination time could be reduced and this may have a positive effect on herd fertility (DeJarnette *et al.*, 2004). Reformulation of standard protocols and the formulating of new approaches that alleviate the detrimental effects of cryopreservation on sperm function are two methods of attempting to improve the fertility of cryopreserved semen. However, a requirement of both is a better understanding of the overall cryopreservation process, as well as the evaluation of methods used to determine the effects of cryopreservation. Another possible area of research is the identification of seminal fluid components, which may play a role in decreasing the occurrence of cryopreservation-induced capacitation of sperm (Medeiros *et al*., 2002).

Continuous research in the chemotherapeutic control of micro-organisms is also of some importance. The principal reason for this is the fact that these organisms can possibly develop a resistance to antibiotics presently used in semen extenders. As some of these organisms may be able to survive the cryopreservation process, the

effectiveness of antibiotics used is of the utmost importance (Berndtson & Foote, 1976).

2.3 Certified Semen Services (CSS)

Certified Semen Services (CSS) is a sub-division of the American organisation NAAB (National Association of Animal Breeders). The purpose of CSS is to regulate semen collection procedures and animal health management of participating AI collection centres. This is to ensure that the industry is provided with good quality, disease-free semen that is properly identified (DeJarnette *et al.*, 2004; Mitchell & Doak, 2004).

According to CSS guidelines, each sire from which semen is collected, must be tested for 6 diseases at least twice a year. However most semen collection centres exceed these minimum requirements (DeJarnette *et al.*, 2004).

2.4. Advantages and disadvantages of AI and the cryopreservation of semen

Some factors affecting the success rate of AI generally include herd management (including the nutritional status of the females), seasonal effects on reproduction and the quality of semen used. These factors should be managed and controlled in such a way that the advantages of AI application within the herd will be fully utilised (Haugan et al., 2005).

The most obvious advantages of applying AI in a herd include the control of venereal diseases, as well as reduced costs. When compared to other reproductive technologies, it becomes apparent that AI is a very simple, yet successful and economical method that can be applied to introduce new genes in a population (Vishwanath, 2003).

The presence of lethal genes can also be reduced and genetic improvement of milk production traits by the selection of bulls used in AI programs can be seen as benefits on their own. However they also collectively lead to overall economic benefits for the dairy industry (Foote, 1999). Sperm cryopreservation is then also used to build fertility reserves for endangered species (Ehmcke & Schlatt, 2008).

The ability to control the transmission of venereal diseases possibly had a larger effect on the initial decision to apply AI commercially than the genetic advantages.

However careful and continuous monitoring of the health status of donor sires, and regular disease testing of their semen is of critical importance. If this is not done efficiently, AI can become a very effective tool for transmitting diseases to herds worldwide (DeJarnette *et al.*, 2004).

Much of the labour and money saved by not having to keep bulls on site may have to be spent in the detection of cows in oestrus and the restraint on these cows for AI. As dairy cows are confined for milking each day, some of these costs are more easily justified in a dairy, compared to a beef farm (Salisbury *et al.,* 1978). Another limitation of semen cryopreservation is that only mature sperm can be preserved, and thus sperm from pre-pubertal males cannot be preserved with any degree of success (Ehmcke & Schlatt, 2008). As semen quality and fertility tends to decline with age, it is advantageous to preserve semen from younger bulls. Semen from young, sexually inactive males can also be preserved while awaiting the progeny test results (Foote, 1975).

2.5. Economic implications of AI in cattle

Effects resulting from the commercial implementation of AI, are most prominent in the dairy cattle industry. The overall result of implementing AI is accelerated genetic progress, resulting in improved production capabilities of the animals (Medeiros *et al.,* 2002). Improving the production efficiency of dairy herds has the benefit of satisfying both the producer and consumer. The increased demand for inexpensive products of acceptable quality is satisfied, and a higher profit potential for producers is generated. (DeJarnette *et al.*, 2004)

Depending on the reason why AI or semen cryopreservation is implemented, the economic implications of these procedures will differ. The various reasons for preservation of male fertility may lead to different approaches, depending on the discipline of interest (Ehmcke & Schlatt, 2008).

2.6. Characteristics of the sperm cell

Sperm have certain characteristics that give them the distinction of being ‘terminal cells’. They are as such, very susceptible to injury and are usually the preferred cell used in research into optimal cryopreservation procedures. Some of the characteristics of sperm include being haploid and containing very little cytoplasm

and other organelles. Chromosomes within the nucleus are very condensed, which prevents protein transcription taking place. The minimal quantity of endoplasmic reticulum present on the Golgi apparatus is not sufficient to maintain membrane integrity, therefore sperm have to depend on the absorption of surrounding molecules to supply the necessary building blocks to maintain cell membranes and undergo maturational changes (Medeiros *et al.,* 2002).

2.6.1. Capacitation and acrosome reaction

Ejaculated sperm of mammalian species as such are not ready to fertilize an oocyte immediately, and have to be in the female reproductive tract for a period of time. This time period is species-specific, and is regulated by the female's hormone status in order to prevent gamete ageing. In 1951, Austin and Chang independently discovered that sperm have to undergo a process called capacitation within the female reproductive tract, before it could fertilize an oocyte. It was determined that the acrosome reaction could only take place if capacitation had already occurred, and it became standard practice to use the acrosome reaction as an endpoint in determining whether sperm were fully capacitated (Medeiros *et al.,* 2002; Curry, 2000).

Initially capacitation was defined as all structural and maturational changes of the sperm, within both the male and female reproductive tracts. Presently these changes are categorized into two main events: capacitation *per se*, and the acrosome reaction. Changes during capacitation cannot be observed at the structural level, but several of these changes have been characterized at the molecular level. The overall effect of the capacitation process is that essential processes collectively known as the acrosome reaction can be activated, when a spermatozoon interacts with an oocyte. Once the capacitation process is activated, the lifespan of sperm is limited. It has also been determined that – compared to fresh semen – cryopreserved sperm had a shorter lifespan after insemination, but that cryopreserved sperm penetrated oocytes much faster than fresh sperm.

Changes that occur during capacitation include the inactivation of decapacitation factors present on the surface of the sperm and adsorption of proteins from the female tract onto sperm. As well as changes in the localization, lateral mobility and

molecular structure of integral proteins and changes in the lipid composition of cell membranes.

In contrast to sperm capacitation, the biochemical processes of the acrosome reaction are well-defined at the structural level. The acrosome reaction processes include the formation of fusion points between the plasma and outer acrosomal membrane, followed by membrane vesiculation – then the acrosomal enzymatic contents are extruded and the inner acrosomal membrane antigens exposed (Medeiros *et al.,* 2002; Curry, 2000).

The acrosomal caps of sperm undergo a distinct sequence of changes as these cells age or are damaged. The rate of these changes depends on individual bulls, ejaculates and handling procedures of semen after collection. There is also a good correlation between fertility in the field and the rate of change (Robbins et al., 1976).

2.7. Principles in semen cryopreservation

There are certain principal factors that should be kept in mind during the process of extending and cooling of semen. These factors will affect sperm independently, but many interactions between these factors also affect the sperm. Many of these interactions have been studied, which have led to combining different procedures. The result was the development of 'systems' for semen preservation (Saacke, 1983). Some of these factors involved ambient temperature, energy source, osmotic pressure and electrolyte balance, buffering and pH, proper gas phase, inhibition of microorganism growth, exclusion of toxic materials, the 'dilution' effect and protection of sperm against the cold (Salisbury *et al*., 1978).

2.7.1. Temperature

As the metabolic rate of cells are generally proportional to the absolute temperature, semen is kept at lowered temperatures (usually 5°C) during storage, as this slows down the chemical reactions and thus extends the lifespan. It is important to note that the rate of cooling semen to 5°C must be controlled to prevent the occurrence of cold shock. Metabolic rate is not the only factor affected by a lower temperature – as the temperature decreases, both the internal and external environments of cells undergo changes. So for instance gas solubility for environmental gases increase as the temperatures decreases. At decreased

temperatures a large part of the total metabolic activities are due to oxidative metabolism – because of the air present and increased solubility for gases at these temperatures (Salisbury *et al*., 1978; Bearden *et al.,* 2004).

2.7.2. Energy source

Sperm do not only require energy for motility, but also for cell maintenance. Sperm can utilize energy sources through both aerobic and anaerobic metabolism, as the energy source naturally available in seminal plasma is fructose – diluted greatly during semen extension. Thus a supplementary source of energy such as glucose or fructose should be added to the extender. It is known that egg yolk contains several compounds suitable for use as energy sources by bovine sperm (Salisbury *et al*., 1978).

2.7.3. Osmotic pressure and electrolyte balance

Sperm are capable of adapting in size according to the tonicity of different media. Also cell permeability differs for various substances. Although sperm are tolerant towards the tonicity of media, if this deviates too much it could result in sperm having bent tails, swimming in circles and ultimately dying. Solutions with compositions similar to that of body fluids, like egg yolk and milk, seem to be the most compatible with sperm (Salisbury *et al*., 1978; Bearden *et al.,* 2004).

2.7.4. Buffering and pH

Sperm need to be protected from auto-toxication from acids that build up as a product of the metabolism. This effect is more pronounced if semen is not kept at a lower temperature, which decreases the rate of metabolism. Extenders containing egg yolk or milk usually have a pH of approximately 7, but it may even be beneficial to reduce the pH to 6.5 (Salisbury *et al*., 1978; Bearden *et al.,* 2004).

2.7.5. Proper gas phase

As the cells' permeability for gases increase with decreased temperatures, the proportion of gas liquid phase, as well as the composition of the gas phase should be controlled to maintain the desired gas conditions within the extender.
Care must also be taken to limit exposure to light; as such exposure in the presence of oxygen damages the sperm cells. Gassing with nitrogen will be beneficial to the cell (Salisbury *et al*., 1978).

2.7.6. Inhibition of microbial growth

While there are many positive aspects to the use of natural products such as egg yolk and milk as additives, there are also downsides to its use, as part of the extending media. These products provide a fertile breeding ground for microorganisms, which produce many products harmful to sperm, or that have the potential to infect the cows. This is one of the reasons why it is standard procedure for antibiotics to be included in any medium used for the extension of bovine semen (Salisbury *et al*., 1978).

2.7.7. Exclusion of toxic substances

Any extender should be prepared to be free of any and all harmful substances like e.g. heavy metals. These substances should also prevent the formation of any toxic products during storage. Even though care is taken to minimize exposure to toxic products, some products, such as glycerol or dead sperm, cannot be totally eliminated. Glycerol, a cryoprotective agent that protects cells during freezing,can be toxic to sperm at high levels. Dead sperm also provide amino acid oxidase, which produces H_2O_2 – a toxic substance to sperm (Salisbury *et al*., 1978).

2.7.8. Semen extension and the “Dilution” effect

Semen should be extended in such a way that the initial sperm concentration is reduced to a number that yields optimum results. It has been shown that dilution (or extension) lowers the motility of sperm, but that the addition of certain amino acids and macromolecules, such as egg albumin or casein, minimizes this effect. Rottensten *et al.* found that if egg yolk or milk was included in the extender, the effect on sperm motility was almost prevented (Salisbury *et al*., 1978).

2.7.9. Protection of sperm against cold shock

Sperm are subjected to cold shock when they are cooled down to 5°C, and this causes intracellular enzymes and other material of the cell to leak out, as well as decreased motility. Cold shock also then causes the flagella of sperm to bend. It is not known what the exact mechanisms of damage are, but the presumed explanation is that temperature changes occur at different rates both internally and on the surface of the cell that results in both physical and chemical damage. Lecithin, proteins, lipoproteins, milk and egg yolk have proved to be the most effective agents in preventing cold shock (Salisbury *et al*., 1978).

The application of consistent, stringent quality control standards is essential for all semen suppliers to supply the best possible semen to breeders. For this reason, individual batches of semen should always be frozen using the best known cryopreservation procedures (Foote, 1975).

2.8. Fresh versus frozen semen

Successful cryopreservation of animal semen has the potential to enhance the advantages of applying AI further, and even provide additional advantages of its own. However, in order to achieve this, the results have to be at least comparable to that of fresh semen. (Bailey et al., 2000)

In order to achieve success when applying AI with frozen semen, the damaging effects on the cells caused by freezing procedures must be reduced to a minimum. This is generally a complex problem, as these effects can occur during any of the procedures of semen extension, equilibration and freezing. Therefore, all procedures should be kept as simple and straightforward as possible. Cryopreservation of bovine sperm is a good example of the potential success that can be achieved with cryopreserved semen (Amirat *et al.* 2005; Curry, 2000).

Even though there has been considerable progress in semen cryopreservation; both the viability and fertility of the sperm still deteriorate during the cryopreservation process. This deterioration is a result of numerous cellular injuries occurring throughout the process. These injuries have to be minimised, in order to improve the survival rate of sperm, which could thus result in higher fertility rates being obtained. (Medeiros *et al.,* 2002)

Many researchers have reported a post thaw motility of 50% of a fresh semen sample motility to be acceptable. This has been achieved in a number of species. Regardless, the number of sperm needed per successful insemination is still variable between species. In some species, the fertility rates of cryopreserved semen can be comparable to that of fresh semen – if the number of sperm per insemination is high enough (Curry, 2000; Medeiros *et al.,* 2002; Chaveiro *et al.*, 2006). In 1995 Shannon and Vishwanath reported that to achieve fertilization rates in cattle comparable to that of fresh semen, the number of cryopreserved sperm needed per insemination was 8 times more than the required number for fresh semen AI (Bailey et al., 2000). When Shannon (1978) determined the number of

fresh and frozen bovine sperm needed per insemination to yield comparable fertility rates in the 1970's, it was clearly demonstrated that 10 times more frozen sperm were required to yield the same result as a dose of fresh sperm. Although the total number of sperm required per fresh and frozen insemination dose has decreased from 2.5 million and 25 million in 1978 to 1-1.5 million and 10-15 million respectively (Vishwanath, 2003) – the fresh: frozen sperm ratio still remained 1 to 10 (Holt, 1997).

Mattner *et al.* (1969) reported a poor *in vivo* survival rate of sperm that were not irreversibly damaged by the cryopreservation process, as a possible reason for the vast difference in sperm numbers required for fresh and frozen semen AI. Watson (1995) suggested an accelerated capacitation process characteristic of cryopreserved sperm (Holt, 1997). When either fresh or cryopreserved semen is used with AI, insemination times as well as site of insemination and number of sperm per dose should be adapted to obtain maximum fertility (Medeiros *et al.,* 2002).

Female animals are usually inseminated when they are observed in oestrus. As ovulation can occur at different times during oestrus, insemination usually occurs either before or after ovulation – but very rarely during ovulation. If the inseminated semen consists of a heterogeneous sperm population – as has been proven to be the case with fresh semen. The probability of fertilization improves drastically because the population consists of sperm in different stages of development. This means that there will almost constantly be a sub-population of sperm ready to fertilize the oocyte during the period of oestrus. It has been demonstrated that cryopreservation of semen drastically reduces the heterogeneity of the population. This may be yet another explanation for the lower fertility rates achieved with cryopreserved sperm, compared to fresh semen (Curry, 2000; Januškauskas & Žilinskas, 2002).

2.8.1. Effect of cryopreservation on bovine sperm

It is a well-known fact that freezing of sperm causes structural damage. This is a major reason why progress in developing methods for the cryopreservation of sperm was slow. In 1979 Jones and Stewart found the cooling of sperm to 5°C only to cause the acrosomal head to swell. However the freezing and thawing of

sperm caused the acrosome to rupture as well as causing damage to the middle piece in a large number of sperm. Cryopreserved sperm display signs of being in an advanced stage of capacitation after thawing, even though these sperm were only at the onset of capacitation, prior to the freezing process (Medeiros *et al*., 2002).

The biggest problem encountered when freezing any cellular structure is the phase changes that all membranes and cell contents undergo. As the temperature lowers, eventually the liquid phase changes into a solid phase and this causes structural changes. As the liquids transform into solids, ice or crystals, which can cut through membranes, form. As the external solvents change from a liquid to solid phase, the concentrations of other solutes increase dramatically in the remaining liquid. The entire cell must then respond to all these changes in the limited time allowed by the protocol used. During the thawing process, the cell again has to go through a reverse process, where the solid phase changes back into a liquid phase. All of the above mentioned changes underline the importance of identifying a rate of temperature change that allows water and cryoprotectant movement – without causing intracellular crystal formation or any irreversible membrane changes. (Hammerstedt *et al.,* 1990; Bailey et al., 2000; Januškauskas & Žilinskas, 2002)

There exist two main causes of sperm cell volume changes occurring during the process of cryopreservation. The first is the effect of glycerol when added to cells in an isotonic medium. This causes an initial rapid shrinking stage followed by a slower return to the original volume, as glycerol penetrates the sperm cells. The second cause of volume changes is the freezing of the extracellular water. This results in an outward water movement because of the high extracellular salt concentration caused by freezing. When sperm cells are thawed, these processes are reversed. It is also important to remember that there are species differences regarding the freedom with which water can move within the sperm. This can have a significant effect on the cryopreservation of sperm in different species. (Hammerstedt *et al.,* 1990; Bailey et al., 2000)

Several factors that seem to play a role in the success of cryopreservation of semen of different species have been studied. Drobnis *et al.,* (1993) found that

species with lower cholesterol levels present in the cell membranes (including bovine and ovine) were more susceptible to membrane damage during cryopreservation. White (1993) concluded that sperm from species with a higher ratio of unsaturated to saturated fatty acids (bull and ram), are more sensitive to lower temperatures. Another possible factor is the reactive oxygen species (ROS). ROS is thought to play a role in the initiation of the capacitation process (Bailey et al., 2000; Medeiros *et al*., 2002; Griffin, 2004).

As sperm cells are not adapted to endure extreme temperatures, many of these sperm are injured during the cryopreservation process. There are different mechanisms during each of the phases, resulting in different injuries to the cells. The sperm injuries can be divided into two main groups: sperm injuries occurring during cooling and due to cold shock, and cellular injuries inflicted during freezing.

The whole practice of bovine semen processing can essentially be divided into 5 steps. Step 1 is the extension of semen and cooling to 5°C. Step 2 includes the addition and equilibration period of glycerol, as well as the packaging. Step 3 is the freezing, step 4 the semen storage and step 5 the thawing process. Each of these steps has specific effects on the sperm cell membrane and metabolism. So for example step 1 causes changes in the physical properties of the cell membrane due to temperature changes. Step 2 causes a large change in cell volume to which cells have to adapt while Step 3 causes further modifications to both the sperm cell volume and membrane structures in a very limited period of time. Step 4 represents a dormancy period of the cell and step 5 requires the sperm cell membranes to recover from changes caused by previous steps – to expand back to normal. (Dunn *et al.*, 1953; Hammerstedt *et al.* 1990; Medeiros *et al*., 2002)

Between the ejaculation of semen and fertilization of an oocyte there are however several phases where man can influence the ability of sperm to successfully fertilise an oocyte. These phases include the evaluation of initial semen quality, extension and cooling to 5°C, glycerolation, equilibration period, packaging, freezing procedure, storage and thawing (Foote, 1975).

2.8.2. Effect of cold shock on bovine semen

The phenomenon known as 'Cold Shock' could be explained as the irreparable damage to several areas of an individual spermatozoon and it can be observed as a permanent loss of motility in the hawed sperm. Cold shock usually takes place when the semen temperature rapidly decreases from approximately body temperature of the bull (35°C), to the freezing temperature of water (0°C) (Blackshaw & Salisbury, 1957; Medeiros et al., 2002; Bailey et al., 2000).

Several researchers have found that cold shock can be prevented by slowly cooling semen to 0°C over a few hours. Here egg yolk can act as a protective agent, when added to semen prior to cooling (Blackshaw & Salisbury, 1957).

Some of the physical damage resulting from cold shock include damaged sperm cell membranes and altered metabolic functioning - possibly also caused by modifications in membrane components' arrangement. These changes occur during a thermotropic phase transition, when the phospholipids convert from a liquid-crystalline to gel phase and results in a firm membrane structure. Several membrane elements influence the damage resulting from cold shock. Some of these elements include the cholesterol/phospholipid ratio, protein/phospholipid ratio, degree of hydrocarbon chain saturation, as well as the non-bilayer-preferring lipid content. However, preventative measures to avoid the occurrence of cold shock do exist. These include controlling the rate of cooling and the addition of protective agents to the semen extenders. Research determined the optimum rate of cooling (from body temperature to 5°C) to be -10°C/hour, and that using either egg yolk or milk as protective agents result in the minimal occurrence of cold shock (Medeiros et al., 2002).

Even in the presence of a cryoprotectant such as glycerol, the cryopreservation process is essentially still a moderate version of cold shock. Only a portion of the original sperm population survives the cryopreservation process and is still capable of fertilizing oocytes. The rest of the sperm cells are reversibly or irreversibly damaged by the process (Bailey et al., 2000).

2.8.3. Effects of low temperatures on bovine semen

Decreasing the temperature below 5°C creates a new set of complications. As the temperature approaches -10°C, there is a high risk of ice crystal formation as the intracellular water freezes. These crystals can cause mechanical stress on the cellular structures and ultimately lead to disruption within these structures. Mechanical stress caused by ice formation occurring around cells also have a damaging effect on cells, as the cells are confined to very small spaces of unfrozen solutes.

When temperature is between -5 and -10°C, ice formation occurs within extracellular fluid – while the intracellular fluids remain supercooled. In order to maintain chemical equilibrium between intra- and extracellular fluids, cellular dehydration occurs. During this period, the rate of cooling is critical, it has to be slow enough to allow sufficient cellular dehydration, but also fast enough to freeze the remaining intracellular fluid, before exposing cells to the hyperosmotic conditions resulting from dehydration (Medeiros et al., 2002).

2.9. Pre- and post-freezing evaluation of bovine semen

By simply improving AI and semen cryopreservation techniques, the minimum number of sperm needed per dose can be decreased, while still yielding optimal fertility results. Methods of semen evaluation become essential in order to evaluate any potential improvements in these techniques (Saacke, 1983). A successful commercial evaluation method must be fast, easy and relatively inexpensive (Januškauskas & Žilinskas, 2002).

Fertility data of AI bulls gathered from information of semen used in the field, is of very little value as it excludes any samples rejected by laboratory evaluations used to identify semen suitable for freezing and subsequent use for AI. (Woods *et al.,* 1986)

Evaluating the viability of sperm before, during and after cryopreservation and storage is the foundation of any research relating to semen preservation – because of this, accurate and dependable evaluation methods are critical.
Many of these methods have been questioned during earlier research studies relating to correlations between evaluated factors and fertility which seemed to be inconsistent.

In 1961 Salisbury and VanDemark suggested an asymptotic model as explanation for these apparent inconsistencies. This model explained the relationship between fertility and semen viability to be as follows: as the number of sperm increase towards a threshold value, which may differ between species, as well as individuals of the same species, fertility will also increase. At this threshold value, maximum fertility is reached, which means that further increases in sperm numbers will not result in increased fertility. This implies that if semen samples used in a study to observe the correlation between fertility and a specific trait surpass the threshold value for that specific quality trait, no correlation between fertility and the specific trait exists. However if the semen samples contained sperm below the threshold value, the conclusion would be that a high correlation existsbetween the trait and fertility. Several studies done by researchers have supported this model (Saacke, 1983).

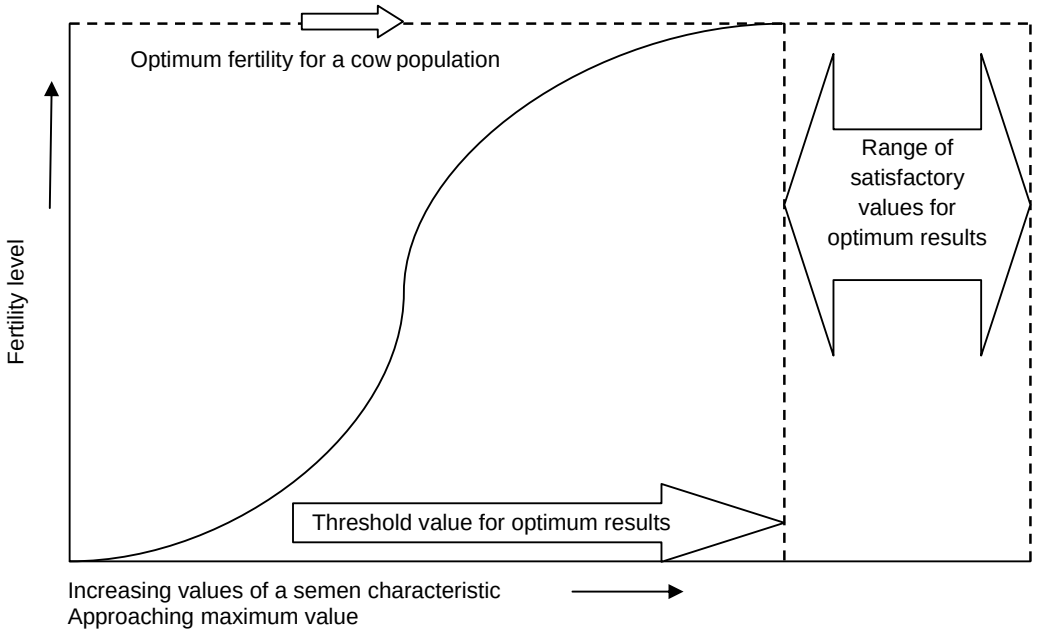


FIGURE 2.1: Fertility increases in response to increasing values of semen characteristics until a threshold value is reached. After the threshold is reached, fertility no longer increases in response to increasing semen characteristic values. (Adapted from Salisbury *et al.*, 1978)

The ultimate aim of all semen assessments is to identify a set of parameters that will accurately predict the fertilizing ability of the semen. When looking from a biological perspective, the only sperm that are potentially fertile, are those

containing intact genetic material. Because of this, the majority of semen evaluation techniques generally concentrate on the DNA integrity and viability of the sperm (Januškauskas & Žilinskas, 2002).

Factors that have an effect on the results of laboratory tests for semen quality include the type of extender, the amount of glycerol included in the extender, the rate of cooling used, equilibration period, packaging and rate of freezing and thawing of the semen (Gaillard & Kupferschmied, 1982).

Generally, semen studies make use of a light microscope to evaluate classic parameters like sperm concentration, motility, morphology and viability. Semen characteristics are classified – according to the likelihood of conception – as either compensable or uncompensable. Compensable traits are defined as parameters whose poor fertility results can be compensated for by increasing the total number of sperm per inseminate. This will result in improved fertility results. Uncompensable traits include all traits resulting in poor fertility that cannot be corrected by increasing the number of sperm per inseminate. As a semen sample can contain both compensable and uncompensable traits, it is important to be able to identify traits that can be compensated for and those that cannot. In only a few decades' semen quality tests have developed from a subjective motility analysis, to much more sophisticated assessment methods such as the analysis of chromatin and membranes – for molecular changes (Januškauskas & Žilinskas, 2002).

Some of the most common techniques used to determine semen quality include the general appearance and volume of the ejaculate, concentration of sperm, the percentage progressive motile sperm, percentage morphologically sound sperm, percentage unstained sperm (using the live-dead stain) and for frozen sperm certain some stress tests (Foote, 1975). Although ejaculate volume and sperm concentration are the classic measurements used to estimate the number of sperm in a semen sample, the currently two most popular parameters evaluated (with the aid of light microscopy) are motility and gross morphology of the sperm. Sperm motility is generally used because of the simplicity of the evaluation method. Graham *et al.* (1956) reported a positive, but non-significant correlation between fertility and motility rate as measured by microscopic evaluations. Even

though sperm motility is only an indicator of flagellar activity and thus does not guarantee fertility of the sperm, it is still of importance as the sperm have to be motile in order to make contact with the oocyte. This motility can then be evaluated as quantitative and qualitative motility. Quantitative motility generally includes the percentage sperm demonstrating progressive motility, while qualitative sperm motility consists of the speed of movement, sperm movement patterns, as well as the degree of head displacement. These tests are all subjective and depend on the ability and experience of the technician.

Sperm morphology is another parameter commonly used to evaluate the quality of the sperm, although semen pH and sperm metabolism can also be used (Salisbury *et al.*, 1978; Foote, 1999, 2002; Januškauskas and Žilinskas, 2002). The basis of semen evaluation using sperm morphology is the direct relationship between specific morphological defects and the bull's *in vivo* fertilizing capacity. Using sperm morphology as an evaluation criterion, bulls with potentially poor fertility can be identified, before entrance into progeny testing programmes. This can result in major savings for many AI enterprises. As semen assessment using light microscopy is subjective, the use of computer-aided sperm head morphology analysis (ASMA) is becoming more popular. Factors such as head length, width, area and perimeter can be evaluated with the aid of ASMA (Januškauskas & Žilinskas, 2002).

Another objective sperm evaluation method available is the CASA (Computer Assisted Semen Analysis) system. This method yields extensive information relating to the kinetic properties of the ejaculate. With the aid of CASA, a correlation has been confirmed between sperm motility characteristics and *in vivo* fertility. Although CASA is considered to be an objective evaluation method, the number of sperm evaluated is limited, which means there is still a small degree of human bias involved . There exist several other laboratory evaluation techniques, including the ATP concentration, SCSA (Sperm Chromatin Structure Assay) and several staining methods used to evaluate the membrane integrity of sperm (Januškauskas & Žilinskas, 2002).

A technique that can be applied when studying the relationship between semen quality and fertility, is called heterospermic insemination. With this technique

differences between inseminators, management factors and biological factors are eliminated. Heterospermic insemination involves the insemination of a female using a mixture of equal number of sperm from two bulls. Paternity of the offspring is then determined and a ratio for the two participating bulls is determined. It was demonstrated that the heterospermic ranking of bulls differed when fresh and frozen semen was used for insemination. The practical application of this technique is however limited by the number of bulls that can be studied, as well as the fact that mixed semen is undesirable in commercial AI. The time between insemination and determining the paternity of offspring is also a limitation (Saacke, 1983; Foote, 2002).

Although semen quality tests can be used to rank bulls with unknown fertility, these tests are not highly correlated to within bull ejaculates. Different combinations of tests should be researched to determine the combination, with the best prediction of fertility of different ejaculates (Foote, 1975).

2.10. Factors affecting post-thaw sperm motility

There are several factors that can have an effect on the final number of sperm that survive the collection, processing, freezing and thawing procedures. These factors include the initial motility of the sperm, prior to any processing or freezing procedures, the quantity of extender added to the collected semen sample, the freezing curve applied, the method of storage and the thawing procedure. These factors can in turn be influenced by several other factors. Initial sperm motility of the collected semen sample can be affected by the individual bull, the environment, as well as the available semen collection facilities. The extent to which the collected semen sample is diluted during the processing procedures can in turn be influenced by the type of extender used – including the source of cold shock protection, the buffering agent, the amount of glycerol added, the antibiotics added and the pH of the extender. The method in which the extender is added to the semen sample and the period of equilibration also plays a role. The freezing curve used to freeze the extended semen is influenced by the rate of temperature decline, as well as the final temperature at which semen will be stored until thawed. The method of storage may be determined by the packaging of the frozen semen, as well as the period that semen has to be stored.

2.10.1. Effect of initial sperm motility of collected semen

2.10.1.1. Effect of bull (genetics and nutrition) on the semen quality

Several researchers have established the fact that semen collected from individual bulls show significant differences in post-thaw motility regardless of factors like initial semen quality, thawing rate, storage period. Foote (1970) demonstrated the effect of individual bulls on fertility to be much larger than the effects of other factors like the type of extender, glycerolating procedure and motile sperm numbers per insemination. In order to obtain the optimal results for individual bulls, the specific optimal conditions for each individual bulls should be determined (Buch et al. 1956; Bean et al., 1963; Almquist et al., 1979).

Individual bulls show great variation in their ability to produce semen that will freeze successfully. This means that even under identical conditions, semen collected from different bulls will not freeze with the same level of success (O'Dell & Hurst, 1955; Almquist *et al.,* 1979). These differences between individuals can be of either genetic or non-genetic origin, and seem to be consistent between ejaculates. So for example, it has been established that the semen quality of an individual bull may decline with age (Foote, 1975). The poor survival ability of sperm from 'poor freezers' can be compensated for to a limited extend by increasing the number of sperm per insemination dose (Curry, 2000).

Woods et al. (1986) reported a lower correlation between semen characteristics and fertility within bulls compared to between bulls. This implies that predictions will be more reliable regarding bull fertility, compared to the fertility of the individual ejaculates. So, if a bull's true fertility can be established, and appropriate processing procedures are applied, variation between ejaculates become less important. Another factor to consider is the heritability of testicular traits. Testes size has been reported to be an indication of bull fertility. Studies revealed a positive correlation between bull fertility and testicular size. Due to the high heritability of testis size, more emphasis has been placed on this specific trait during selection and the evaluation of bulls for breeding purposes (Foote, 2002). There exists a correlation between these traits and semen quality, as well as fertility. More emphasis however must be placed on both the initial pedigree selection of the bulls and also performance testing of the bulls at puberty (Foote,

1975, 2002). After the selection of bulls with the potential to produce superior semen, management of these animals as well as proper processing and handling procedures of the collected semen become the next critical factors (Foote, 1975).

2.10.1.2. Effect of the environment (season and temperature) on bull semen quality

In the process of domesticating cattle, animals were selected to avoid seasonal breeding. This means that females should be able to ovulate and also conceive at any time of the year. In spite of this, some breeds are still susceptible to factors causing seasonal breeding. These factors include photoperiod, temperature, humidity and nutrition. It has been established that the semen quality of bulls can also be affected by season (Haugan et al., 2005). Other factors that can detrimentally affect thawed semen, include sunlight and the presence of oxygen. Packaged semen kept at 5°C has shown decreased sperm motility after exposure to radiation (Coulter & Foote, 1977).

2.10.1.3. Effect of collection procedure on the post-thaw sperm motility of bovine semen (facilities, handler, method and number of ejaculates)

Good facilities are essential during the semen collection procedure – whether the artificial vagina (AV) or electro-ejaculator method is used. The AV-method is currently the most commonly used method worldwide in cattle. Semen is collected with the aid of a rubber funnel, enclosed in a jacket filled with warm water (approximately 42-44°C at the time of collection). Semen collection is performed while the bull is mounting a teaser animal. The temperature and pressure of the artificial vagina (when used) is of some importance, as these also acts as stimuli to initiate ejaculation (Mitchell & Doak, 2004; Salisbury *et al.*, 1978).

Semen can also be collected using the electro-ejaculation method. This method is usually reserved for bulls unable to mount teaser animals or that are not used to being handled. A bipolar probe is inserted into the rectum of the bull. This probe is used to send alternating electrical currents to nerves surrounding the accessory glands and base of the penis. This will result in ejaculation of the semen (Mitchell & Doak, 2004; Salisbury *et al.*, 1978).

When comparing the electro-ejaculation to the artificial vagina method of collection, Austin *et al.* (1961) found the electro-ejaculator to result in larger, but less concentrated volumes of semen. Although semen collected with an electro-ejaculator is less concentrated than semen from the artificial vagina, the larger volumes generally result in about the same number of sperm per collection.

Other methods of semen collection include massaging the sex accessory glands of the bull or collecting semen from the vagina of the female after natural mating. These methods are not commonly used, as contamination of the ejaculate commonly occurs.

It is essential to collect the maximum quantity of semen from bulls during each collection, in order to meet the high demands for semen from superior bulls. Research on improving ejaculates was done earlier and sexual preparation is used to increase the volume, as well as the sperm density per ejaculation. Almquist *et al.* and Bratton *et al.* investigated the effect of sexual stimulation prior to semen collection, as well as the intensity of each collection. These studies have resulted in the recognition of the important role that sexual responses of individual bulls play in the quality of the semen collected. It was also suggested that these stimuli should be adapted for each individual bull in order to achieve optimal ejaculation results. This is usually done by allowing the bull to mount a teaser animal a few times before collection. Teaser animals used are usually other bulls or steers. Cows can also be used as mount animals, but this is not recommended. `A collection frequency of 6 times per week was suggested. The volume of sperm collected is also greatly affected by the frequency of ejaculation (Foote, 1975, 2002; Salisbury *et al.*, 1978; Mitchell & Doak, 2004).

2.10.2. The effects of dilution on post-thaw sperm motility of sperm

The two most important functions of any semen extender are to preserve fertility and increase the semen total volume, for convenient handling. In a normal fresh bull ejaculate, the sperm concentration is normally very high. In order to get a semen volume suitable for convenient insemination, without wasting sperm, extension or dilution of the ejaculate is necessary. Many supplements maintaining and protecting the sperm may then also be added during extension.

Initially, the media used to dilute semen was comprised mainly of salt or sugar solutions, and the goal was not to conserve, but rather to increase the volume for immediate use. All this however has changed when the preservation value of egg yolk was discovered. This discovery, together with the knowledge that the metabolic rate of sperm was reduced at 5°C meant that semen could be preserved for considerably longer periods of times (Salisbury *et al.*, 1978).

2.10.2.1. Effects of the type of extender on dilution rate

The word “extender” has replaced the original term “diluent”, as extenders actively extend the life of sperm and there is a negative connotation to the word “diluent” (Salisbury *et al.*, 1978; Foote, 2002).

The initial purpose of an extender was to prolong the life of sperm, to allow for transport of the semen. Today this is still the main goal of a successful extender, but other requirements have been added – such as increasing the volume of a single ejaculate so that it can be used to inseminate several female animals (Mitchell & Doak, 2004). After the development of an effective freezing method, the next big challenge was to develop a medium that could protect the sperm cells from the extreme environmental changes occurring during cryopreservation (Bialy et al., 1957).

The first major discovery with regard to semen extenders, was in 1940 when Phillips and Lardy reported the protective properties of egg-yolk when used as part of a yolk-phosphate extender. A year later Salisbury *et al.* improved on this extender by replacing the phosphate component with sodium citrate. This resulted in a clearer medium, which allowed for better microscopic evaluation of the extended semen. Later on antibiotics and glycerol were also added to the extender to prevent the transmission of venereal diseases and improve the survival rate of the sperm after cryopreservation. Milk was found to be a useful replacement for egg-yolk, but egg yolk is currently still the most common ingredient used (Foote, 2002; DeJarnette *et al.*, 2004).

The main challenges faced when preparing semen extenders, are total quality control, as well as the control of non-pathogenic environmental contamination. When using non-pasteurized egg yolk, the risk of microbial contamination is also

implicated. The use of pasteurized and microbiologically monitored egg yolk has been suggested, but the best solution would be the use of a substitute of non-animal origin that is pathogen–free (Van Wagtendonk-de Leeuw *et al*., 2000). Attempts have also been made to develop a completely synthetic medium that can be used in bovine semen processing. However egg yolk and whole milk extenders still remain far superior to any of the tested synthetic models (Foote, 1999).

Semen extender or diluent preparation takes place in the laboratory environment, usually in an AI laboratory. It is a labour intensive process, and human error is a critical factor. This generally means that each batch of prepared extender is of a slightly different quality. From this perspective, industrial preparation, with proper quality control and less handling could be a better option. In an experiment comparing commercially produced vs. locally prepared extenders, showed both extenders to yield similar results. (Van Wagtendonk-de Leeuw *et al*., 2000)

There are significant interactions between the extender used and freezing method, as well as extender and storage or incubation temperature. This should be taken into account whenever any one of these factors is changed (Bean et al. 1963). Because of the interaction between different extenders and processing procedures, a change in one step during processing will possibly affect all the other steps. Some of the factors to be kept in mind include the initial semen quality (ejaculate should be mixed with extender in a gentle, but thorough manner; to ensure uniform distribution), equilibration and cooling of the semen (extender should always be added to semen before cooling) and glycerol equilibration (levels of glycerol added will vary between extenders and freezing methods – when glycerol is added at 5°C, the rate of supplementation is not significant) (Foote, 1975).

Some of the requirements suggested for any successful semen extender include ensuring isotonic conditions, providing adequate buffering capacity and energy, and the extender containing a plasma-membrane protecting agent (Pesch & Hoffmann, 2007).

2.10.2.1.1. Different protectants utilised against cold shock (egg yolk, milk and soya)

After Phillips and Lardy discovered the benefits of including egg yolk in semen extenders in the 1940's, other researchers started investigating the protective qualities of egg yolk. These early researchers confirmed that egg yolk improved the ability of sperm to resist cold shock. However before detailed investigations could be performed, interest was diverted when Polge *et al.* discovered that bull semen could successfully be frozen with the use of glycerol as cryoprotectant (Pace & Graham, 1974). Blackshaw and Salisbury (1957) reported that egg yolk stimulated glycolysis in unshocked sperm. A study demonstrated that better fertility and sperm motility could be obtained if egg yolk was included in both the initial and glycerated diluents, compared to the then standard procedure of only in the initial diluent containing egg yolk (Sullivan and Mixner, 1963). A study by demonstrated that the beneficial effects of egg yolk during cryopreservation is not easy to reproduce, when considering the number of sperm with intact membranes after thawing (Van Wagtendonk-de Leeuw *et al.,* 2000). Pace and Graham (1974) also found that in the absence of egg yolk neither the semen extender alone or the extender plus glycerol provided adequate protection to the sperm. However when egg yolk was included in the extender, there was a significant improvement in the post-thaw motility of the sperm. This improvement was even more pronounced with the inclusion of both egg yolk and glycerol in the extender. Phospholipids and LDL (low density lipoprotein) components appeared to be crucial components of egg yolk involved in minimizing the occurrence of sperm cold shock. Phospholipids in the egg yolk thus have the ability to lessen the chilling injuries to the sperm, without altering the physical properties or intrinsic membrane composition (Medeiros et al., 2002).

An experiment performed by Foulkes *et al.* (1980) recorded no significant difference in the survival rate of frozen bovine sperm, when diluted with diluents containing egg yolk from hens fed different diets (to obtain egg yolks with different lipid fatty acid compositions). Similarly significant differences in the lipid fatty acid composition of egg yolks from hens fed different diets were obtained but this did not result in any noticeable changes in sperm survival.

Using a citrate-buffered egg yolk semen extender resulted in improved optical clarity compared to the clarity, when using the egg yolk phosphate extender. This citrate-buffered egg yolk and whole milk extenderare the two most common semen extenders used currently (Foote, 1999).
Kampschmidt *et al.* established that egg yolk lipoproteins protect the sperm from cold-shock and can serve as storage media. The yolk phospholipids also provide protection from cold-shock, but were a poor storage media (Bialy et al., 1957).

In a study by Almquist (1954) the fertility results from unfrozen bull semen extended with a yolk-citrate extender was compared to that of unfrozen semen extended with a heated homogenized milk extender. The results showed a significant increase in the fertility rate of semen from bulls with low fertility when a heated homogenized milk extender was used to extend the semen. This increase in fertility however was only significant for semen collected from bulls with low fertility. Perkins *et al.* (1954) also found a tendency for semen from bulls with lower fertility to significantly improve when a milk diluent was used. There was however, no significant difference between the non-return rates for semen collected from bulls with normal fertility, when either a milk or yolk-citrate semen extender was used.

The protective mechanism of skim milk appears to be contained within the protein fraction (Medeiros et al., 2002). Berndtson and Foote (1969) reported that the use of skim milk as a replacement for egg yolk resulted in unsatisfactory post-thaw sperm survival rates, when applying a rapid freezing rate. Certain researchers have found the overall milk to give better post-thaw survival rates, compared to yolk-citrate. However when different freezing methods were compared, yolk-citrate yielded superior results (Bean *et al.,* 1963).

Using ingredients of animal origin in semen extenders has certain disadvantages e.g. the possible negative effect shown in studies with polypeptides in frozen/thawed sperm as origin, and the risk of microbial contamination. These risks can be minimized with including ingredients of plant origin. Soya bean has been identified as a possible alternative to egg yolk (Hinsch et al., 1997).

2.10.2.1.2. Buffering solutions (phosphate, Tris and citrate)

Phillips first reported the advantages of using the egg yolk of the hen as part of a yolk-phosphate semen extender. Salisbury later replaced the phosphate used by Phillips with sodium citrate, and recorded better sperm motility after prolonged storage and importantly, better visibility of the semen sample during microscopic evaluation (Salisbury *et al.*, 1978). Becker *et al.* (1977) recorded a higher sperm motility rate when Tris was included in the extender, compared to the inclusion of citrate in the extender. Berndtson and Foote (1969) when studying the effects of different semen extender ingredients, with varying glycerol levels, found that, especially at lower glycerol levels the Tris-egg yolk extender yielded significantly higher post-thawing motility and sperm survival rates – compared to when only egg yolk was included.

2.10.2.1.3. Cryoprotective agents (glycerol)

The cryoprotective properties of glycerol was discovered in 1940. Glycerol was later classified as a penetrating cryoprotectant. This being due to its ability to penetrate the sperm cell. Both penetrating and non-penetrating cryoprotectants (e.g. lactose) have been shown to have the ability to cause cell dehydration. Thus dehydration is the result of an osmotically induced water flow, out of the cells. The only difference between penetrating and non-penetrating cryoprotectants is their ability to enter the sperm cells. Glycerol also has an intracellular effect by permeating the cell membrane. Thus, glycerol limits the intracellular osmotic stress effect of dehydration, by replacing fluid lost to the extracellular fluids – to maintain the cell volume, interact with ions and macromolecules, as well as lowering the freezing point of the intracellular water. Although there is little known regarding the specific process of cryoprotectant transport into cells, it is expected that this process will occur at a slower rate than that of water transport, when using similar membrane structures. This would mean that any changes in the membrane will affect both the water and glycerol transport. When this is taken into account, the temperature at which glycerol is added to semen becomes very important, as temperature affects the membrane structure. Permeating CPA's (Cryoprotectant Agents) like glycerol may be toxic to sperm cells at high concentrations, by inducing membrane damage and decreasing the motility of the sperm (Hammerstedt *et al.* 1990; Foote, 1999; Curry, 2000; Medeiros *et al.,* 2002).

Berndtson and Foote (1969) observed a decrease in pre-freeze motility of sperm as the level of glycerol in the extender was increased from 0 to 12%. This demonstrated the toxicity of glycerol towards fresh semen. Although a glycerol concentration of 10% of the extender volume is generally accepted as too high (thus having a toxic effect on bovine sperm), interactions between the cooling rate, glycerol concentration and thawing rate can affect the level at which glycerol will negatively affect the sperm. The toxicity level of glycerol n semen also differs between species. Robbins *et al.* (1975) found that in order to attain optimal motility of sperm following cryopreservation, the level of glycerol should be increased when rapid thawing rates are used. It was also reported glycerol levels should be decreased when using rapid freezing rates. This illustrates the fine balance that must be maintained with the inclusion of glycerol in a semen extender (Becker *et al*., 1977; Curry, 2000; Chaveiro *et al.*, 2006). Although glycerol levels as high as 15% have been recommended for the optimal efficiency of bovine semen cryopreservation, generally glycerol levels of 7 to 8% are used by commercial bull semen processing organisations (Salhab & Merilan, 1991).

Saroff and Mixner (1954) found a significant interaction between the levels of egg yolk and glycerol present in the diluents. A theory was suggested that egg yolk somehow ‘tied up’ the glycerol, as results demonstrated a higher glycerol requirement linked to the increasing egg yolk levels. However compared to previous experiments – where egg yolk was present only in initial semen diluents, this ‘tying up’ effect could also be linked to equilibration time. As in this specific experiment egg yolk was present in both the initial and glycerol-containing diluents.

When a Tris-egg yolk semen extender was used, no differences were recorded in either the motility or fertility of the sperm – whether glycerol was included in the extender initially or added slowly after cooling to 5°C (Coulter & Foote, 1977). Foote (1975) stated that the temperature at which glycerol addition takes place can be significant, depending on the extender used. With some extenders glycerol can be added with the initial extension of semen (at room temperature), without adversely affecting the sperm. However when a milk-based or yolk-citrate extender is used, glycerol should not be added at room temperature.

TABLE 2.1: A summary of studies on different glycerol equilibration periods

Equilibration Periods:	**Results:**	**Authors:**
1, 2, 4, 8 and 16 h	2h optimum, >4h not advised	Wiggin & Almquist (1974)
2, 6, 12 and 18 h	Motility increase with GET	Saroff & Mixner (1954)
2, 6, 12 and 18 h	2h inferior to 6, 12 & 18h ($p<0.01$)	Sullivan & Mixner (1963)
4,10, 16, 22 and 28 h	14.9 h optimum – as GET decrease, %survival decrease slowly	Cragle *et al.* (1954)
10s, 30 min and 6 h	10s significantly higher motility compared to 30min, 6 h	Berndtson & Foote (1969)
0 and 2 h	2 h increased PTM ($p<0.01$)	Dhami & Sahni (1993)
4 and 18 h	18 h ($p<0.01$)	Foote & Kaproth (2002)
30 min and 18 h	30 min significantly higher ($p<0.05$)	O'Dell & Hurst (1955)
30 min and 18 h – ram semen	30 min higher – NOT significant	Hill, Godley & Hurst (1959)
4, 8 and 12 h	12 h significantly higher ($p<0.05$) than 4 h, but not significantly higher than 8 h	Graham, Erickson & Bayley (1956)
30 s, 2, 5, 15 and 75 min	No significant difference	Almlid & Johnson (1988)
30 min and 18 h	18 h higher motility – significantly so with fructose	Martin & Emmens (1961)
2, 4, 6, 10 and 18 h	Optimal GET ranged 2-10 h – depending on cooling time (slow cooling needs less GET)	Ennen, *et al.* (1976)
2, 4 and 6 h	4 h optimum	Tuli *et al.,* (1981)
6, 8, 12 and 18 h	12 h superior ($p<0.05$) to other treatments	Roussel, Kellgren & Patrick (1964)

GET Glycerol Equilibration Time
PTM Post-Thaw Motility

2.10.2.1.4. Antibiotics

According to Salisbury *et al.* (1978) Gunsalas *et al.* were the first to draw attention to the number of bacteria present in collected semen samples. Generally less than 150 000 organisms are present in semen samples, but this can range from zero to several million in a specific sample (Salisbury *et al.*, 1978). After the reports on the effect of bacterial control it is now standard procedure to include several antibiotics in any semen extender used in the cattle AI industry. An increase in fertility was also observed when adding antibiotics to the extender. Mainly as the growth of bacteria causing venereal diseases e.g. Vibriosis, was inhibited by the use of antibiotics. The addition of antibiotics allowed for sperm preservation at room temperature, without excessive bacterial growth. There are currently several precautions to be taken during semen collection, to limit bacterial infection. Some of these include sterile equipment and cleaning the sheath and underline of the bull before semen collection (Foote, 1999).

The best solution to prevent the spreading of disease through cryopreserved semen is still the maintenance of pathogen-free bulls. As this is not always possible, the addition of antibiotics to the semen has become a standard practice. This is done to control the micro-organisms that contaminate the semen during the collection and processing procedures (Berndtson & Foote, 1976). Some of the most common antibiotics included in semen extenders include sulfanilamide, penicillin and streptomycin (Berndtson & Foote, 1976; Salisbury *et al.*, 1978; Foote, 2002).

2.10.2.1.5. pH of the semen extender

In order to prevent autotoxication – resulting from glycolysis, it is imperative to keep the pH of extended semen neutral (6.5 to 6.9) (Griffin, 2004). Bean *et al.* (1963) found significant differences in the pH of yolk-citrate and milk based semen extenders, when comparing them before and after freezing – as well as after different storage intervals. Within each extender, however, there were no significant differences regarding the pH after different freezing protocols, storage temperatures and intervals.

2.10.2.2. Method of extender addition to semen (dilution methodology – 1 step or 2 step)

Jeyendran and Graham (1980) reported that there was no significant difference in the progressive semen motility when cryoprotective agents were added at either 35 or 5°C. Similarly Foote (1970) found that fertility was similar whether glycerol was added in one step or slowly in 4 steps. Proving that the slow addition of glycerol was unnecessary.

2.10.2.3. Equilibration intervals of extended semen

As mentioned earlier, the presence of glycerol is potentially toxic to fresh semen. Equilibration periods with glycerol should thus be balanced, in order to utilize the cryoprotective properties of glycerol – without any unnecessary loss of sperm prior to cryopreservation (Berndtson & Foote, 1969; Foote, 1975).

The subject of optimal glycerol equilibration time has long been the debate among experts in the field of cryobiology. The glycerol equilibration period is generally viewed as the period that cells are left in a glycerolated semen extender medium prior to cryopreservation (O'Dell and Hurst, 1955; Leite *et al.,* 2010).

Several studies have reported that both post-thaw motility of sperm and fertility were improved when semen was allowed to equilibrate for several hoursusing glycerol. This suggested that considerable time was needed for the protective action of glycerol to take an effect. However, other studies have suggested that a longer 'aging' period at 5°C, would result in improved sperm survivability and fertility, regardless of the period of glycerol equilibration employed (Berndtson & Foote, 1969; Foote & Kaproth, 2002).

In a trial performed by Sullivan and Mixner (1963), it was found that no significant interaction between the different semen samples and glycerol equilibration time occurred when testing different techniques of egg yolk addition and glycerol equilibration times. This is in contrast to the significant interaction that O'Dell and Hurst (1955) obtained when using only sperm motility while Sullivan and Mixner (1963) used various methods of evaluation.

Berndtson and Foote (1969) found that a 10 second glycerol equilibration period resulted in significantly higher sperm motility, compared to motility resulting from

either a 30 minute of 6 h equilibration period. From this it was concluded that the harmful effects of glycerol took effect within the first 30 minutes of equilibration. Two possible explanations for the superior motility results with a 10 second equilibration were offered. The first possibility was that even though glycerol fully penetrated the sperm, there was not enough time for the negative actions of glycerol to take place – this theory was based on the assumption that complete penetration of sperm cells is necessary for the protective properties of glycerol to take effect. The second possibility was that the short equilibration period only allowed for partial penetration of the cells, which actually enhanced the protective effect of glycerol. The original glycerol equilibration time for egg yolk diluents, was 15 to 20 h, but others have proposed a 6 h equilibration period to be adequate to achieve optimum sperm survival (Sullivan and Mixner, 1963; O'Dell and Hurst, 1955).

In an experiment performed on 6 Holstein bulls in the 1950's, a general tendency for higher non-return rates was obtained with longer equilibration times. It was reported that glycerol equilibration periods of 20 to 24 h were optimal (Graham *et al.,* 1956). An experiment by Saroff and Mixner (1954) tested 2, 6, 12 and 18 h equilibration intervals and confirmed increased sperm survival with longer equilibration periods. However higher non-return rates resulting from inseminations with semen equilibrated for 6 h rather than 18 h were reported (Graham *et al.,* 1956). It was also found that 6 h of equilibration was sufficient for optimal survival of sperm. Cragle *et al*. (1955) performed a series of experiments to determine the optimal glycerol equilibration period and proposed a 14.9 h equilibration period to be optimal.

O'Dell and Hurst (1955), using an egg-yolk based extender found a 0.5-h equilibration period to be more favourable, when compared to an 18-h period. In contrast, Martin and Emmens (1961) found an 18 h equilibration period to result in more favourable fertility results, compared to a 30 minute equilibration period, when including 1.25% fructose in the extender. When Hill *et al.* (1959) studied the sperm motility of ram semen after glycerol equilibration periods of 0.5 or 18 h, no significant difference between the resulting percentages were recorded.

Gilbert and Almquist (1978) studied the effect of glycerol equilibration periods (0, 3 and 9 h), and found significantly lower sperm motility, as well as increased retained acrosomes, with no equilibration period compared to equilibration periods of either 3 or 9 h. A study by Dhami and Sahni (1993) found a 2 h equilibration period to be superior to no equilibration period, when post-thaw sperm motility and fertility rates were used as criteria.

Salhab and Merilan (1991) studying different glycerol equilibration periods for bovine semen, found a 2 h period to yield superior results, compared to a 4 h equilibration period. In the study by Leite *et al.* (2010), no equilibration period resulted in significantly lower post-thaw sperm motility, compared to equilibration periods of either 2 or 4 h. Ennen e*t al.* (1976) studied different combinations of cooling and glycerol equilibration periods and found that optimal post-thawing motility was obtained when combining the cooling periods of 2 or 4 h with glycerol equilibration periods of 4 or 10 and 2 or 4 h, respectively.

A 4 hour equilibration period was found to be superior to equilibration periods of either 3 or 6 h for semen collected from buffalo bulls (Tuli *et al.,* 1981).

A possible explanation stated for the contrasting results regarding glycerol equilibration periods, may be the application of different freezing rates (Polge, 1957).

2.10.3. Effect of the freezing curve on post-thaw motility of bovine sperm

Most researchers have found that when undiluted bovine semen is rapidly cooled, there is a dramatic decrease in sperm survival, and therefore a slower rate of cooling was recommended (Foote and Bratton, 1949). When comparing the results of semen samples extended before and after initial cooling, it showed that pre-extended samples recorded a much higher percentage of motile sperm, than post-extended samples. Anderson and Seath (1948) demonstrated that any delay in cooling, and especially when extending a freshly collected semen sample, results in decreased sperm keeping qualities. It has been recommended that semen should be extender as soon as possible after collection (Foote and Bratton, 1949).

By decreasing the temperature to 5°C, cellular metabolic activity of the sperm cell is decreased, which in turn extends its life span. This expansion however is limited, as sperm mainly use catabolic processes to function, and has very limited biosynthetic activity. Eventually the sperm's metabolic activity ends in cell death, as the final step in an intrinsic ageing process. Temperature must be reduced to at least -130°C to completely stop all metabolic processes, including thermally driven chemical reactions (Medeiros *et al.*, 2002).

The position and number of semen straws within the freezing unit may also play a role in the variation of the actual freezing curve to which the individual straws are subjected. Another source of variation in the freezing curve may be the lack of uniform conditions to which successive batches of semen are subjected to during the freezing process (Foote, 1975; Robbins et al., 1976).

2.10.3.1. Rate of temperature decline (Cooling rate)

The optimal cooling rate will depend on the semen extender, as well as the packaging used. There exist some interaction between the extender used and the cooling, as well as thawing rate. A good guideline has said to be -15°C/min from +5°C to -100°C, then transferring the semen to liquid nitrogen for storage (Senger *et al.*, 1983; Foote, 1999). In order to minimize the risk of cold shock, semen is thus cooled at a slow rate to a holding temperature of approximately 5°C, and kept at this temperature for a period. This is to allow the cell membrane structures to alter and adjust. These changes in membrane structure also allow for the use of a drastically higher cooling rate, without adversely affecting the cells (Chaveiro *et al.*, 2006).

The relationship between sperm cell survival and the cooling rate after ice nucleation for bovine semen can be seen as having the shape of an inverted 'U'. This implies that the superior rate will permit the fastest freezing rate for the extracellular fluids, without causing ice formation within the intracellular fluids. With too low cooling rates there is an increase in the level of dehydration and cell shrinkage - thus low sperm cell survival. Too high cooling rates also cause low sperm survival, because it causes intracellular ice formation, which is generally lethal to the cells. Optimal freezing rates for bulls are reported to be between 50 and 100°C/min (Medeiros et al., 2002; Chaveiro *et al.*, 2006).

2.10.3.2. End temperature of freezing curve

Initially cryopreserved semen was stored in solid carbon dioxide – also known as 'Dry Ice®' – at a temperature of -79°C. However in the 1950's there was a shift toward using liquid nitrogen with a lower temperature (-196°C). Studies have proven the storage in liquid nitrogen to be acceptable for any length of time without affecting the post-thaw qualities of the sperm. With storage at -79°C certain biological changes still occurred, which limited the length of time in which the sperm could be stored in solid carbon dioxide. Carbon dioxide also has to be refilled more frequently than liquid nitrogen. When liquid nitrogen was first introduced, storage tanks were not sufficiently insulated to avoid leakage, but this has been improved. Today liquid nitrogen containers are much improved and form an essential basic part of the modern AI industry (Foote, 2002).

It is however important to avoid semen being partly thawed while being stored – even at temperatures as low as -70°C, and then being allowed to cool again to an end temperature of -196°C. This usually occurs with poor semen handling and storage techniques and is possibly the most damaging action, when considering the viability of sperm after cryopreservation (DeJarnette et al., 2004).

When bovine semen stored at temperatures of -79°C, -92°C or -196°C were evaluated for motility, semen stored at -79°C recorded significantly lower sperm motility ($p<0.01$), compared to semen stored at either -92°C or -196°C (Bean et al. 1963).

2.10.4. Effects of storage technique on the post-thaw motility of bovine sperm

2.10.4.1. Packaging of frozen semen

Bovine sperm can be frozen and stored in plastic straws more efficiently than in 1ml glass ampules. A comparison of sperm frozen in plastic straws vs. glass ampules showed more favourable results regarding the fertility of sperm frozen in straws (Rodriguez *et al.,* 1975). The superiority of using a straw freezing system compared to an ampule system was demonstrated when employing a yolk-glycerol-citrate based extender (Saacke, 1983). It has also been established that the 0.25-ml straw to be superior to the 0.5-ml straw, but the processing and freezing techniques should also be adapted for the use of either of these types of

straws. Using the 0.25-ml straw allows for more efficient storage and thus reduced costs (Senger *et al.*, 1983). Semen packaged in straws are generally exposed to a mainly nitrogen environment, while semen packaged in glass ampules are exposed to more atmospheric gases (Coulter & Foote, 1977).

To achieve optimal results, all handling techniques and cryopreservation processing procedures should be adapted for the use of plastic straws, instead of glass ampules. There is a greater surface-to-volume ratio when using straws, compared to ampules, and this results in sperm being more responsive to any thermal changes. However despite the increase in responsiveness of sperm, the sperm can still tolerate a relatively wide spectrum of freezing rates, without being damaged significantly. It appears as if the optimal glycerol level for sperm frozen in straws decreases as the freezing rate increases. The glycerol level should thus be adapted for the different methods of freezing. Storage units thus have a major effect on factors such as the optimum freezing and thawing rate, as well as the optimum level of glycerol included in the extender (Rodriguez *et al.,* 1975; Robbins *et al.,* 1976). A study by Rodriguez *et al.* (1975) showed semen frozen in straws placed in a horizontal position to have a slightly higher survival rate, compared to straws frozen in a vertical position.

2.10.4.2. Period of storage of frozen bovine semen

No difference in fertility was observed by Graham *et al.* (1956), when studying different times of storage (1, 2 or 3 weeks). Sullivan and Mixner (1963) also recorded no significant effect when storage times of 2 weeks, 3 months and 6 months were investigated. However, according to O'Dell and Hurst (1955), there was a significant decrease in the sperm survival rate for samples left in storage for 10 days, compared to samples for only 48 h. It was subsequently recommended that samples only be evaluated after 10 days, in order to obtain more accurate results within a reasonable period of time.

The correct handling of cryopreserved semen is critical to limit the damage to semen viability. Exposing semen to the ambient temperature for even a few seconds, can be harmful and have an affect the final post-thaw motility of the sperm. Semen frozen and maintained constantly at -196°C did not show any decline in the post-thaw motility or fertility after a period of up to 24 months. Some

success has even been achieved with semen stored for 12 years (Foote, 1975). Coulter and Foote (1977) found a significant decline in sperm motility stored for 6 months, compared to a storage period of 0 months.

2.10.5. Method of thawing frozen bovine semen

Bovine semen can be thawed using various methods and rates, in either air or water. The rate of thawing should be high, with the final temperature being no higher than 38°C. A few methods worth mentioning include air thawing (Bean, 1972), thawing in the cow (Davidovic *et al.,* 1971), rolling the semen straw between the palms of the hands (Macpherson & Penner, 1972) and warm water thawing (Aamdal & Anderson, 1968; Robbins *et al*., 1972 and 1973; Almquist & Wiggins, 1973). The latter method is currently the most popular method used commercially (Rodriguez *et al.* 1975; Foote, 1975,1999).

When thawing with warm water, excessive warming must be prevented as cold shock can occur during subsequent exposure to the ambient temperature. As the temperature in the neck of the liquid nitrogen containers is much higher than -196°C recommended, the end temperature after thawing may be higher than expected and cold shock can occur with exposure to the ambient temperature. Thus thawing times should be kept to a minimum, but still ensure complete thawing (Rodriguez *et al.,* 1975). The optimal thawing rate is generally determined by the interaction between the processing factors like e.g. glycerol level, freezing rate and method of packaging. Therefore it is advised that thawing recommendations of specific manufacturers are followed (Foote, 1975; DeJarnette et al., 2004).

Studies have shown that post-thaw bovine sperm motility and acrosome maintenance are increased when the semen packaged in straws, are thawed rapidly. However these thawing rates should be accurately timed, to avoid higher internal straw temperatures which may lead to sperm damage (Becker *et al*., 1977). It has been recognised many times that the rate of semen thawing should be adjusted for different freezing rates – to ensure optimum survival rates of sperm. (Hammerstedt *et al.,* 1990)

Data collected by O'Dell and Hurst (1955) demonstrated that thawed semen lost motility more rapidly than fresh semen, and thus must be utilized on the day of thawing only, for best results.

Cryopreserved bovine semen should be thawed as close to insemination time as possible – as the lifespan of these sperm is much shorter than that of fresh semen. It is also recommended that straws should be thawed a maximum of 15 minutes before AI. Semen handling techniques up until insemination are also of importance (DeJarnette et al., 2004).

Several researchers have found a longer thawing period to be favourable, compared to the shorter thawing periods (Gaillard & Kupferschmied, 1982; Almquist et al., 1979, 1982b).

It is then important to follow the correct protocol when thawing frozen semen. Although thawing semen at 70°C for 5 seconds was superior to thawing at 37°C for 20 seconds, it is more practical to use the latter temperature commercially. It is of the utmost importance that thawed semen not be exposed to a temperature below 15°C (Pesch & Hoffmann, 2007).

CHAPTER 3
MATERIALS AND METHOD

3.1. Introduction

3.1.1. Aim of study

The aim of this study was to determine the consequences of altering standard equilibration periods recommended for the semen extender used during processing of semen from African cattle breeds. Several combinations of cooling and glycerol equilibration periods were evaluated and compared using post-thaw spermatozoa motility as an indicator of cryopreservation success.

The null hypothesis for both Experiment 1 and Experiment 2 was that there would be no significant differences in post-thaw motility percentages or recovery fractions (fraction of pre-freeze progressively motile sperm surviving cryopreservation) of spermatozoa between the different cooling and equilibration periods. Thus the alternative hypothesis for both experiments was that there would be significant differences in post-thaw motility percentages or recovery fractions of spermatozoa between the different cooling and equilibration periods.

3.2. Experimental design

The research was subdivided into two experiments. Each experiment researched different cooling and glycerol equilibration times as well as different combinations thereof. The first experiment (referred to as ‘Experiment 1’) involved shorter cooling times of 30, 60, 120 and 240 minutes respectively, with each cooling time followed by several longer equilibration periods of 4, 5, 6, 7 and 8 h. In the second experiment (referred to as ‘Experiment 2’) the cooling and equilibration times of the first experiment were reversed. This resulted in longer cooling times of 4, 5, 6, 7 and 8 h respectively, with each cooling time having shorter glycerol equilibration periods of 30, 60, 120 and 240 minutes. The experimental designs of Experiment 1 and 2 are presented in Figures 4.1 and 4.2.

FIGURE 3.1: Flow Diagram for Experiment 1

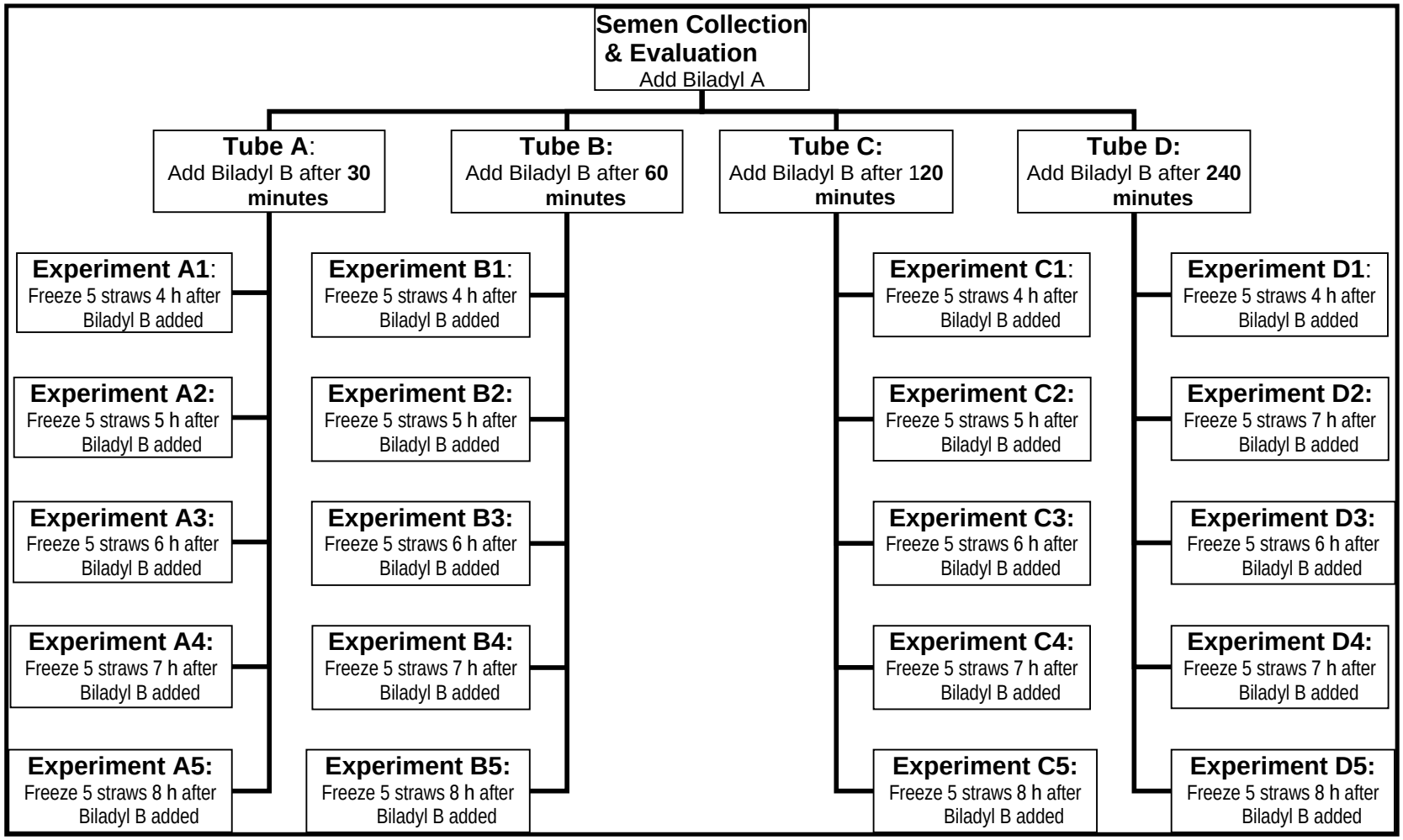
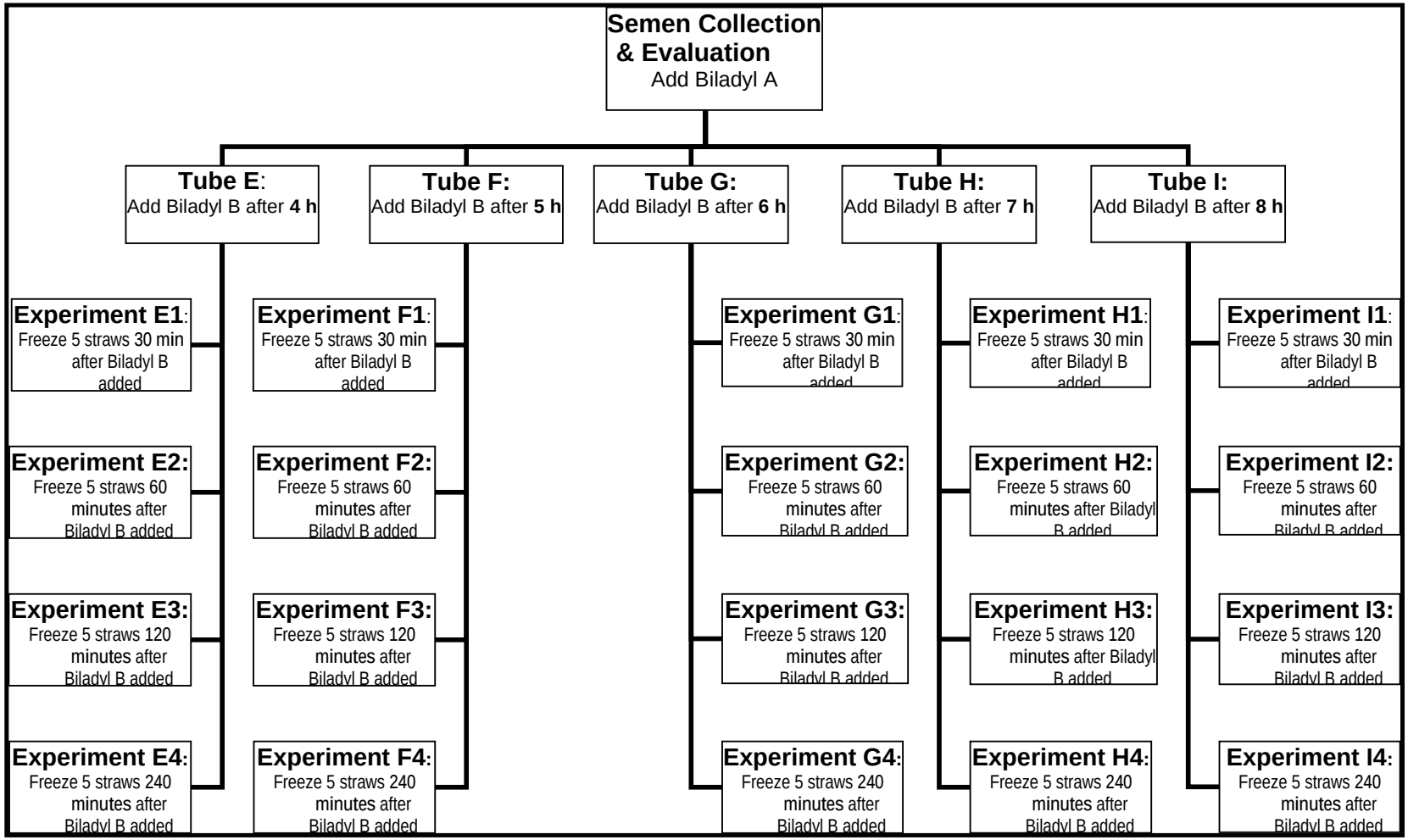


FIGURE 3.2: Flow Diagram for Experiment 2



3.2.1. Nutrition

All bulls were fed a standard daily diet consisting of 2kg 12% protein concentrate pellets and 10kg dried Lucerne (*Medicago sativa*). Eragrostis (*Eragrostis curvula*) hay bales and fresh water were always available *ad libitum*.

3.2.2. Housing

All bulls were housed at Embryo Plus, a well-known quarantine station located in the North West Province of the Republic of South Africa. The exact coordinates for the quarantine station are S25°38'27.3'' and E027°47'16.9''. The climate in the area is classified as sub-tropical and the annual rainfall in the area averages 658.89 mm, with an average ambient temperatures fluctuating between 10.58°C and 26.81°C. The average humidity in the area ranges from 29.50% to 80.68% (ARC-Institute for Soil, Climate and Water, Pretoria, 2006).

3.2.3. Ambient temperatures during the experiments

Ambient temperatures varied from a minimum of 8.8°C to a maximum of 31.2°C during the experimental period. Temperature in the laboratory where evaluation and processing took place, was more controlled but still varied within the range of 16.8 to 26.6°C. Environmental temperatures during the experiments were included as independent variables in the statistical model, but the effects thereof on the variables studied (Post-thaw Sperm Motility and Recovery Fraction) were negligible, while the climatic conditions in the laboratory were maintained relatively constant.

Haugan *et al.* (2005) reported that semen collected during the summer months resulted in a significantly higher calving rate compared to semen collected during other seasons. When the environmental temperature rises above body temperature (38°C), in can cause spermatogenesis in bulls to cease, but no evidence was found to suggest that low environmental temperatures had any negative effects on the fertility of the bulls (Bearden *et al.,* 2004).

3.3. Semen extender used for the cryopreservation of bovine semen

3.3.1. Extender components

The semen extender used in these experiments was Biladyl® (Minitüb Abfüll- und Labortechnik GmbH & Co. KG, Tiefenbach, Germany), which is a commercially available Tris-based two-step extender meeting all CSS (Certified Semen Services) requirements. The CSS again forms part of a bigger American organisation, the NAAB (National Association of Animal Breeders). The ultimate aim of the CSS is to develop and maintain guidelines and health testing protocols, resulting in accurately identified and disease-free processed semen. It is important to mention that processed semen from non-CSS laboratories will not necessarily distribute diseases. It only means that there is no guarantee regarding the safety when using the semen.

3.3.1.1. Biladyl®

The extender Biladyl® is packaged as a complete kit, that includes concentrates of the two fractions, as well as the antibiotic cocktail to be added. The concentrates for both fractions are transparent liquids with Tris, citric acid, sugars and buffers dissolved in pure water. Glycerol is also included in the transparent liquid - Fraction B.

3.3.1.1.1. Antibiotic cocktail

The antibiotics used included Tylosin (50 µg/ml), Gentamicine (250µg/ml), Spectinomycin (300µg/ml) and Lincomycin (150µg/ml) as prescribed by the Certified Semen Services (CSS). These antibiotics are included to effectively control the growth of Mycoplasmas, Ureaplasmas, *Haemophilus somnus* and *Campylobacter fetus* subsp. *venerealis* (Griffin, 2004).

3.3.1.1.2. Fraction A

Fraction A represents the non-glycerolated portion, consisting of the concentrate, egg yolk and the antibiotic cocktail.

The concentrate for Fraction A is a transparent liquid with Tris, citric acid, sugars and buffers dissolved in pure water. The buffers implemented in Biladyl include

Tris hydroxymethyl aminomethane (commonly referred to as Tris) and citric acid. Sugars included in the concentrate will be used by the spermatozoa as an energy source. The pure water acts as the diluent, which serves as a transport medium for other extender components, as well as increasing the volume of individual doses for more convenient handling and storage (Peters & Ball, 1987).

3.3.1.1.3. Fraction B

Fraction B consisted of the concentrate (containing 14% glycerol) and egg yolk. The only difference in composition of the concentrates for Fraction A and B was that glycerol was included in Fraction B and not in Fraction A. Glycerol is included as a cryoprotectant to protect spermatozoa during the cryopreservation process.

TABLE 3.1: Summary of extender ingredients (adapted from Griffin, 2004)

Component	Fraction A	Fraction B	Final % of Extender	
Distilled Water	341ml	150ml	49,1%	(491ml)
CSS Antibiotics	10ml	-	1%	(10ml)
CSS Bovine Buffer Concentrate	49ml	180ml	22,9%	(229ml)
Egg Yolk	100ml	100ml	20%	(200ml)
Glycerol	-	70ml	7%	(70 ml)
Total Volume for Fractions A and B	**500ml**	**500ml**	**100%**	**(1000ml)**

3.3.1.2. Egg Yolk

All the chicken eggs used as a source of egg yolk were bought from a local supermarket and refrigerated at 7°C, until used. Fresh eggs were bought the day before the extender was to be prepared. All eggshells were sprayed with 70% alcohol and wiped clean with a paper towel to remove any micro-organisms and other potential contaminants.

3.3.1.3. Water

The quality of water used to prepare the semen extenders can have an enormous impact on semen quality (Minitüb Abfüll- und Labortechnik GmbH & Co. KG, Tiefenbach, Germany). Therefore, it is important that water used in the extender

preparation should be distilled and deionised to remove most bacteria and harmful ions. Sterilization of water is also recommended. Purified water should meet minimum requirements including conductivity below 1µS/cm, less than 1 CFU (Colony Forming Unit) per 10ml and a Total Organic Carbon (TOC) content of less than 50ppb (Minitüb Abfüll- und Labortechnik GmbH & Co. KG, Tiefenbach, Germany). The water used in the extender preparation in this study was deionised and sterilized by using the Modulab® Water Purification, as well as the Modulab® UFV Bio-Ultra filtration Water Systems (U.S. Filter Corporation, Dubai, United Arab Emirates).

3.3.2. Preparation of the semen extender:

In order to dissolve the antibiotics, which are packaged as a white powder stored inside a glass vial, 12ml sterile bi-distilled water was added to the powder with the aid of a sterile syringe. The powder was then given time to dissolve completely.

To ensure that the working surface was sterile it was cleaned with 70% alcohol before use. All instruments used also had to be sterilised (See Appendix A, Figure 2.8). Eggs were sprayed with 70% alcohol and individually wiped with clean paper towels. This was done in order to ensure that no dirt or micro-organisms were present, that could contaminate the extender. After the egg shell was broken by lightly tapping the egg against the side of an egg divider. Egg yolks were obtained by using a sterile egg divider and then transferred to a clean filter. The egg yolk was then carefully rolled around on the filter so that all the albumin could be absorbed. When no more albumin was present on the yolk membrane, the yolk was rolled to the edge of the filter. A sterile needle was used to carefully puncture the yolk membrane and the egg yolk was poured into an Ehrlen-Meyer flask (See Appendix A, Figure 2.9). Care was taken that the yolk membrane or any albumin was not accidentally added to the yolk in the flask. This procedure was repeated for each egg, until the flask contained 100ml egg yolk.

The concentrate of Fraction A was then transferred into a sterile measuring cylinder (See Appendix A, Figure 2.10). The cylinder was filled up to the 390ml mark with micro-filtrated deionised sterile water. This solution was transferred to a sterile

500ml Ehrlen-Meyer flask, together with a sterile magnet and placed on a magnetic stirrer. The stirrer was switched on and the 100ml egg yolk slowly added. Care was taken to prevent foam formation.

Using a sterile syringe, 10ml of the dissolved antibiotic cocktail was added into the flask without causing foam formation. The solution was left on the magnetic stirrer until all the egg yolk had dissolved – approximately 30 minutes (See Appendix A, Figure 2.11).

The Fraction A part of the extender was then transferred into labelled 50ml Falcon tubes, and refrigerated at approximately 5°C.

When mixing Fraction B, 100ml of egg yolk was again collected using the same procedure as described for Fraction A. The concentrate for Fraction B was transferred into a measuring cylinder. Micro-filtrated deionised sterile water was used to fill the cylinder to 400ml. This solution was then transferred to a sterile 500ml Ehrlen-Meyer flask, together with a sterile magnet and placed on a magnetic stirrer. The stirrer was switched on and the 100ml egg yolk slowly added – care again taken to prevent foam formation. The solution was left on the magnetic stirrer until all the egg yolk dissolved – approximately 30 minutes. The Fraction B part was then transferred into labelled 50ml Falcon tubes and stored in a freezer. Prior to use both Fractions were allowed to warm to 37°C.

Both fractions of the extender were prepared in the same week that semen was to be collected and frozen. Fraction A was stored in a refridgerator at 5°C, and Fraction B stored in a freezer at -7°C. Before use, Fraction A was put in a water bath and allowed to warm to 35°C. This ensured that cold shock to the spermatozoa was prevented, as no significant temperature variation between the semen and the Fraction A-extender existed, when mixed (Mitchell & Doak, 2004; Foote, 1978; Salisbury e*t al.*, 1978). Fraction B was then removed from the freezer, placed in the laboratory and allowed to thaw and warm to room temperature before use.

3.4. Bovine semen collection

3.4.1. Introduction

In both experiments the electro-ejaculator was used for semen collection (See Appendix A, Figure 2.12). Essentially this method entails inserting a bipolar probe into the rectum of the bull, connecting the probe to a control unit and stimulating erection and/or ejaculation through electrical stimulation of nerves surrounding the accessory glands and base of the penis. (Salisbury *et al.*, 1978) This method was used because the majority of bulls at the quarantine station came from extensive cattle farms, and the bulls were therefore not halter-trained. In order to make use of the artificial vagina method, bulls have to be trained. All collections were done by the same technician, at approximately the same time of day and employing the same technique.

3.4.2. Number of ejaculates and frequency of semen collections

Ejaculates were collected from Nguni and Boran bulls. For Experiment 1, 9 Nguni and 6 Boran bulls were used, and for Experiment 2, 5 bulls of each breed were used. The first and second ejaculates collected on the day of the experiment were used in the trials. All collections took place between 8:00 and 9:00 on the day of the relevant experiments. No ejaculates were collected from bulls for at least 3 days prior to the day of the relevant experiments. All semen samples used in the experiments were collected by the same technician.

3.4.3. Age and history of the bulls

Bulls from two indigenous breeds – the Nguni (n=9) and Boran breeds (n=6), were used in the study. Both of these breeds are indigenous to Africa, the Nguni being classified as *Bos indicus* Sanga type, sometimes also referred to as *Bos taurus africanus* (Gertenbach and Kars, 1999) while the Boran is classified as *Bos indicus (Zebu-type)* (Maiga, 2006).

Bulls used in the experiment were chosen at random with their ages varying between 2 to 7 years. The total number of bulls (both breeds) included one 2 year old, 8 3 year olds, 4 4 year olds, 1 5 year old and 1 7 year old.

3.5. Preparation for bovine semen collection

Bulls were retrieved from their individual pens and loaded into the crush at least 5 minutes before the semen collection. Bulls were always handled with minimum stress by the same two trained and experienced farm workers

3.5.1. Bovine semen collection procedure

Environmental and laboratory temperatures were recorded at the beginning of each semen collection procedure.

3.5.1.1. Preparations in the laboratory

Before semen could be collected from a bull, preparations in the laboratory, as well as outside, in the collection area, had to be done. In the laboratory the water bath was allowed to warm to 35°C. Sterilized collection tubes and Falcon tubes containing Fraction A of the extender were placed into the water bath and warmed to 35°C. Marking pens used for identifying the different collection tubes were also available. Fraction B of the extender was removed from the freezer and allowed to defrost and warm to room temperature.

The heat-stage of the microscope was warmed to 35°C. The sterile microscope slides, cover slides and a sterilized 1ml syringe were placed on the heat-stage (35°C). It was important that all of the above-mentioned equipment was at the same temperature – as it is a well-known fact that the motility of spermatozoa vary with temperature (Mitchell & Doak, 2004).

A clean cone-holder and sterilized cone were also allowed to warm. A clean container (e.g. small cooler bag), with a sterile 5ml-syringe and needle in the laboratory was allowed to reach room temperature. The container assisted in slowing the cooling rate of the partly-processed semen, and preventing direct contact with the cold air – which could result in cold shock (acting as insulation while semen equilibrated to the refrigerator temperature) (Bearden *et al.* 2004). The partly-diluted semen and a tube of the thawed Fraction B solution were placed inside the container before being placed in the refrigerator. The syringe was used to add the Fraction B to the semen-Fraction A mixture.

3.5.1.2. Preparations in the crush area

The collection area of the crush was cleaned and the floor was covered with clean dry grass to prevent urine, water, dirt or dust from contaminating the semen collection equipment. The following equipment were clean, available and in working order: a scissors, a bucket containing lukewarm water, a bucket containing micro-filtrated sterile water and 2x60ml-syringes, a hand towel, long sleeved rectal AI gloves, liquid paraffin (lubricant) and the electro-ejaculator.

3.5.1.3. Semen collection

When all the preparations were complete, the bull was restrained in a neck clamp and its position secured by inserting poles horizontally behind the hind legs. If necessary, the bull was also immobilised for the sheath wash by using the immobiliser function of the electro-ejaculator.

Before starting with the actual sheath wash, any unwanted long hairs around the prepuce were removed by using a clean pair of scissors. After all hairs were removed, the exterior of the sheath was washed using clean, lukewarm water. This was done to remove any loose hair, dirt, grass and other particles that could potentially contaminate the collected semen sample. After the exterior of the sheath was washed, the 2x60ml-syringes were filled with micro-filtrated sterile water and then used to fill the sheath with the water. The prepuce was kept closed with one hand, while the other hand was used to rub the area between the prepuce and testicles. By rubbing the sheath any urine, soil and hairs that could contaminate the semen sample were removed. After the interior of the sheath was thoroughly cleaned, the prepuce was released and the water allowed to exit the sheath, through the prepuce. After all the water had been squeezed from the sheath, a clean towel was used to dry the underline and prepuce of the bull (See Appendix A, Figure 2.14).

Before inserting the probe into the rectum of the bull, the probe was first lubricated using liquid paraffin. Then one arm, covered by a long sleeved rectal glove, was also lubricated with liquid paraffin and inserted into the rectum of the bull. Any faeces present in the rectum was removed, before the secondary sex glands (vesicular glands) were stimulated by vigorously rubbing it with the open hand through the

rectum wall. In one swift movement the gloved arm was removed, while simultaneously inserting the lubricated probe into the rectum, with the electrodes facing ventrally.

After ensuring that the control unit was switched off and the controlling knob turned to zero, the probe and control unit were connected using the cable supplied. Then the control unit was switched on.

The sterile cone was inserted into the cone holder by folding the wide end of the cone over the cone holder (See Appendix A, Figure 2.13). The collection tube was then connected to the narrow end of the cone. When electro-stimulation began, the cone holder was held in one hand close to the prepuce. With ejaculation, the cone was quickly put in position over the penis to collect the ejaculate (See Appendix A, Figure 2.15).

When stimulating the bull, the controlling knob was initially set to a very low voltage, kept there for 3 seconds and quickly returned to zero. After one second the knob was turned to a slightly higher voltage, for 3 seconds and returned to zero. This process was repeated until the bull ejaculated. Some bulls did not necessarily have an erection before ejaculation, and thus ejaculated into the sheath. In such cases, the ejaculate was collected using one hand to gently rub the outside of the prepuce. Thereby manoeuvring the ejaculate in the direction of the prepuce, while holding the cone in place over the prepuce. Care was taken that the ejaculate was not exposed to direct sunlight at any time as this would harm the spermatozoa. After the ejaculate was collected, the control unit was switched off, the collection tube removed from the cone and the lid screwed on. The collected sample was then taken to the laboratory for evaluation, without being exposed to any direct sunlight.

The collection and evaluation procedures of all semen samples collected during the experiments were performed by the same laboratory technician.

3.6. Bovine semen processing

3.6.1. Evaluation of semen quality

In the laboratory, the collected semen sample was evaluated macroscopically (volume, colour, consistency), as well as microscopically (sperm mass motility, individual sperm motility).

3.6.1.1. Macroscopic (Gross) evaluation

Ejaculate volume was determined using the calibrations on the collection tube. The colour and consistency of the ejaculate were determined subjectively by the technician evaluating the sample. Colour was evaluated by choosing which one of the 4 categories best described the sample. These categories included (from poor to excellent): Grey, White, Ivory and Yellow. Consistency again was categorized into 1 of 5 categories. These categories were Watery (poor), Thin Milk, Milky, Thin Cream and Creamy (excellent).

3.6.1.2. Microscopic evaluation

Individual and mass sperm motility was determined subjectively by evaluating the degree of movement observed using a drop of semen, under a microscope. Mass motility was determined with the aid of an Olympus CH_2 Light Microscope, (10x10 magnification) and individual sperm motility was evaluated, using a magnification of 40x10.

A drop of semen (75µl) was placed on a pre-warmed microscope slide, which was then positioned on the microscope for evaluation, with magnification set at 10x10. Mass motility was evaluated on a scale of 0 to 5. When no mass movement was observed, the sample had an evaluation of zero. As the observed mass movement improved in classification from no movement (zero) to slight (1), linear wave (2), wave (3), heavy wave (4) and whip-like (5) movements, sample classification also improved from 0 to 5 (Mitchell & Doak, 2004).

For evaluation of individual sperm motility, another 75 µl drop of semen was deposited on a sterile microscope slide, and covered with a cover slide (22x22mm). Placing the cover glass over the semen sample ensured that a more uniform smear was available for evaluation. The magnification setting on the microscope was changed to 10x40, in order to observe individual spermatozoal motilityy.

Evaluation of individual motility was scored on a scale of 0 to 100%, with 0% indicating the absence of any linear progressive spermatozoa, and a 100% indicating that all spermatozoa exhibited linear progressiveness (Mitchell & Doak, 2004).

3.6.2. Suitability of semen for cryopreservation

The table as represented in Table 3.2 was used to determine the dilution factor that would yield a total of $15x10^6$ spermatozoa per dose (0.25 ml). The minimum dilution factor of semen destined for cryopreservation was three times - with the evaluation criteria classified as grey, thin milk, 2 and 70%. (See Table 3.2) The dilution factor was increased whenever the result of any evaluation criteria was represented in a column situated to the left of the Minimum Dilution Requirements Column. Each column to the left will cause the dilution factor to increase by 1 unit. Practical application of Table 3.2 is best illustrated, using a hypothetical semen sample. Evaluation criteria for this sample included an Ivory colour, a Creamy consistency, a mass motility of 3 and 80% individual spermatozoal motility. Starting with the minimal 3 times, dilution was increased with 2 units for Ivory, 3 units for Creamy, 1 unit for '3' and 1 unit for 80%. This equates to a 3+2+3+1+1=10 times semen dilution.

Samples with factors that are positioned to the right of the Minimum Dilution Requirements Column in the table are usually not suitable for freezing.

TABLE 3.2: Evaluation Criteria For Collected Semen

FACTOR:				Min. Dilution Requirement (x3)		
Colour	Yellow	Ivory	White	Grey	-	-
Consistency	Creamy	Thin Cream	Milky	Thin Milk	Watery	-
Mass Motility	5	4	3	2	1	0
Individual Motility	100	90	80	70	60	50

(De la Rey, Unpublished data)

3.6.3. Addition of the extender fractions (Experiment 1)

- After semen evaluation was complete and the dilution factor determined according to Table 3.2, the collected semen sample was divided equally into 4 different Falcon tubes (marked Tube A, B, C and D, respectively) using a sterile 5ml-syringe with a needle. It is important to draw attention to the fact that Fraction A was allowed to equilibrate to 35°C, before collection. This was done as a measure to prevent cold shock when extending semen with Fraction A (Foote, 1978; Salisbury e*t al.*, 1978; Griffin, 2004; Mitchell & Doak, 2004).

- The calculated quantity of Fraction A was then added with another sterile syringe to the tubes in amounts calculated according to the dilution factor. To determine the total volume of extender to be added, the volume of the collected semen sample was multiplied by the dilution factor.

- To determine the amount of Fraction A and B to be added to the total collected sample, the total amount of extender was divided by two, as fractions were added in equal volumes.

- The total volume of Fraction A was then divided into 4 parts to determine the quantity of Fraction A that had to be added to each tube. So for example: the collected sample with a volume of 5ml, and the dilution factor was calculated to be 10.

- Then the total amount of extender to be added was (5 X 10) =50ml, and the amount of Fraction A and B (50÷2) =25ml each. The amount of Fraction A and B to be added to one of the tubes would then be (25÷4) =6.25ml for each fraction.

3.6.3.1. Equilibration period with Fraction A ('Cooling Period')

Refer to Table 3.3 for a detailed description of, experimental numbers and the specific combinations of the cooling and glycerol equilibration periods used in Experiment 1.

The period between the additions of the two Fractions was termed the 'Cooling Period' for these experiments. After Fraction A was added to all 4 tubes, the semen-Fraction A mixture was placed inside the insulating container. The Falcon

tube containing Fraction B, a sterile syringe and a needle was also placed into the container. This ensured that all equipment to be used would equilibrate to the changing temperature, at the same rate (Bearden *et al.* 2004).

- The container with its contents was then left to equilibrate at room temperature for 30 minutes.
- 30 minutes after Fraction A was added, the calculated amount of Fraction B was added to Tube A. The container containing the four tubes as well as Fraction B and the syringe was then closed and put into a walk-in fridge (+5°C) to equilibrate further. The rate of cooling from room temperature to fridge temperature was determined as -0.25°C/min. This rate was calculated by measuring the temperature within the container at 10 minute intervals until it reached +5°C. These recorded values were then used to calculate the cooling rate. It took approximately 2 hours for the semen to reach fridge temperature.
- 60 minutes after Fraction A was added, the calculated amount of Fraction B was added to Tube B.
- 120 minutes after Fraction A was added, Fraction B was added to Tube C.
- 240 minutes after the onset of equilibration, Fraction B was added to Tube D.

3.6.3.2. Semen straws used and information recorded

While waiting to add Fraction B to the different tubes, semen straws were identified (printed). For these experiments the French Mini straws (0.25cc), supplied by Minitüb (Minitüb Abfüll- und Labortechnik GmbH & Co. KG, Tiefenbach, Germany) were used.

A different colour straw was used for each bull. Information recorded on each straw included: Experiment Date, Bull Identification Number and specific Experiment Number. A Minitüb EasyCoder Straw Printer (Minitüb Abfüll- und Labortechnik GmbH & Co. KG, Tiefenbach, Germany) was used to print the information on the individual straws. Marked semen straws were also placed in the fridge and allowed to equilibrate to +5°C – in order to prevent a temperature fluctuation when

semen was drawn into the straws. This fluctuation in temperature could have had a negative effect on the viability of the spermatozoa (Bearden *et al.* 2004).

Equipment to be used for cryopreservation was also prepared during this time (See Appendix A, Figure 2.16a and Figure 2.16b). These preparations included logging into the IceCube Series Computer Freezer programme and allowing the freezing chamber to stabilize at 5°C. A polystyrene container filled with liquid nitrogen – this was used to dump straws in liquid nitrogen after straws reached -120°C, during the freezing programme.

3.6.3.3. Equilibration period with Fraction B ('Glycerol Equilibration Period')

Refer to Table 3.3 for a detailed description of Experiment Numbers and the specific combination of cooling and glycerol equilibration periods used in Experiment 1. Also refer to Figure 3.3 for the standard freezing graph used in the freezing process.

The period from addition of Fraction B to the start of the freezing process was termed the 'Glycerol Equilibration Period' for these experiments.

3.6.3.3.1. Cooling Time A (30 minutes)

- 4h after Fraction B was added to Tube A, 5 straws – known as Treatment **A1** – were filled from this tube. The straws were then sealed and frozen, using the IceCube® software programme and equipment (Minitüb Abfüll- und Labortechnik GmbH & Co. KG, Tiefenbach, Germany). When the freezing curve reached -120°C, the straws were removed and immediately plunged into a polystyrene container filled with liquid nitrogen. These straws were then stored in liquid nitrogen, until such time that the straws were thawed for the post-freeze evaluation.

- 5h after Fraction B was added to Tube A, 5 straws – known as **Treatment A2** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.

- 6h after Fraction B was added to Tube A, 5 straws – known as **Treatment A3** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.
- 7h after Fraction B was added to Tube A, 5 straws – known as **Treatment A4** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.
- 8h after Fraction B was added to Tube A, 5 straws – known as **Treatment A5** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.

3.6.3.3.2. Cooling Time B (60 minutes)

- 4h after Fraction B was added to Tube B, 5 straws – known as **Treatment B1** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.
- 5h after Fraction B was added to Tube B, 5 straws – known as **Treatment B2** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.
- 6h after Fraction B was added to Tube B, 5 straws – known as **Treatment B3** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.
- 7h after Fraction B was added to Tube B, 5 straws – known as **Treatment B4** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.
- 8h after Fraction B was added to Tube B, 5 straws – known as **Treatment B5** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.

3.6.3.3.3. Cooling Time C (120 minutes)

- 4h after Fraction B was added to Tube C, 5 straws – known as **Treatment C1** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.
- 5h after Fraction B was added to Tube C, 5 straws – known as **Treatment C2** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.
- 6h after Fraction B was added to Tube C, 5 straws – known as **Treatment C3** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.
- 7h after Fraction B was added to Tube C, 5 straws – known as **Treatment C4** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.
- 8h after Fraction B was added to Tube C, 5 straws – known as **Treatment C5** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.

3.6.3.3.4. Cooling Time D (240 minutes)

- 4h after Fraction B was added to Tube D, 5 straws – known as **Treatment D1** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.
- 5h after Fraction B was added to Tube D, 5 straws – known as **Treatment D2** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.
- 6h after Fraction B was added to Tube D, 5 straws – known as **Treatment D3** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.

- 7h after Fraction B was added to Tube D, 5 straws – known as **Treatment D4** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.
- 8h after Fraction B was added to Tube D, 5 straws – known as **Treatment D5** – were filled from this tube. The same freezing procedure as in Treatment A1 was implemented.

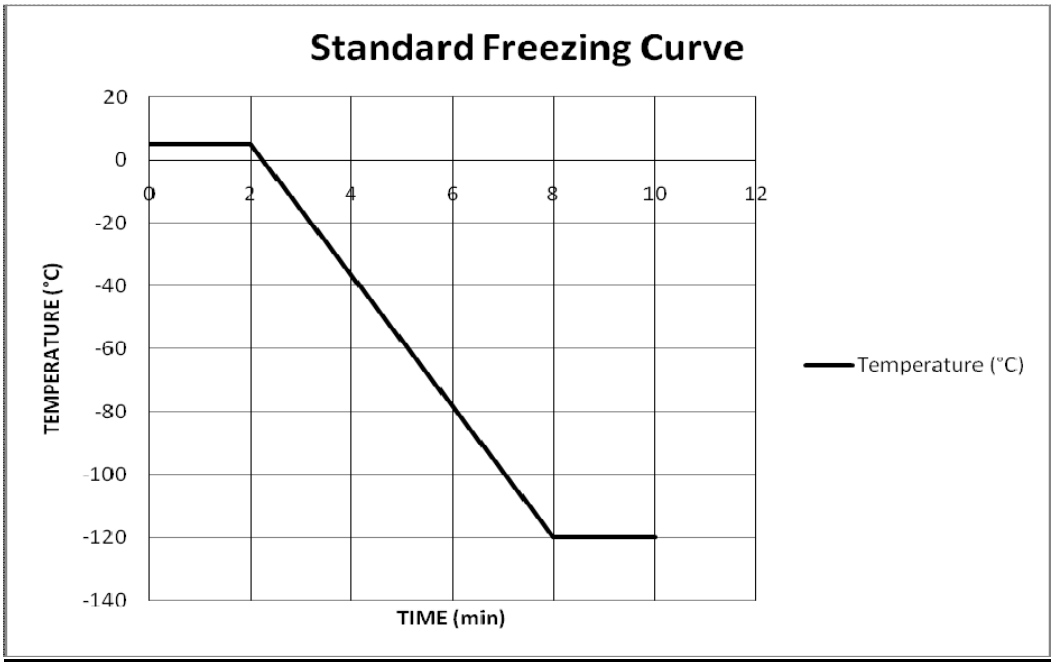
3.6.3.4. Sealing of semen straws for cryopreservation

All straws were sealed using a coloured PVC sealing powder, which transforms into a gel-like substance, when coming into contact with any liquid – such as e.g. the diluted semen. After the semen was drawn into the straws, the open end of each straw was dipped in the powder and sealed by allowing the powder to come into contact with the semen in the straw.

TABLE 3.3: Different experimental numbers used in experiment 1 with the corresponding cooling time and glycerol equilibration time for each number.

	Experiment Number	Cooling Time (hrs)	Glycerol Equilibration Time (hrs)	Total Equilibration Time (hrs)
EXPERIMENT 1	A1	½	4	4½
	A2	½	5	5½
	A3	½	6	6½
	A4	½	7	7½
	A5	½	8	8½
	B1	1	4	5
	B2	1	5	6
	B3	1	6	7
	B4	1	7	8
	B5	1	8	9
	C1	2	4	6
	C2	2	5	7
	C3	2	6	8
	C4	2	7	9
	C5	2	8	10
	D1	4	4	8
	D2	4	5	9
	D3	4	6	10
	D4	4	7	11
	D5	4	8	12

FIGURE 3.3: Standard semen freezing graph used for Experiment 1 and Experiment 2



3.6.4. Addition of semen extender fractions (Experiment 2)

- After semen evaluation was complete and the dilution factor determined according to Table 3.4, the collected semen sample was divided equally into 5 different tubes (marked Tube E, F, G, H and I, respectively) using a sterile 5ml-syringe fitted with a needle. It is important to note that Fraction A was allowed to equilibrate to 35°C, before collection. This was done to prevent cold shock when extending semen with Fraction A (Foote, 1978; Salisbury *et al.*, 1978; Griffin, 2004; Mitchell & Doak, 2004).
- Fraction A of the extender was then added with another sterile syringe to the tubes in the volume calculated according to the dilution factor. To determine the total volume of extender to be added, the volume of the collected semen sample was multiplied by the dilution factor.
- To determine the volume of Fraction A and B to be added to the total semen sample the total volume of extender was divided by 2, as the fractions were added in equal volumes.

The total amount of Fraction A was then divided by 5, to determine the volume of Fraction A that had to be added to each tube. So for example: if the collected semen sample had a volume of 5ml, and the dilution factor was calculated to be 10, then the total volume of extender to be added was (5 X 10) =50ml, and the volume of Fraction A and B (50÷2) =25ml each. The volume of Fraction A and B to be added to each of the tubes would then be (25÷5) =5ml of each fraction.

3.6.4.1. Equilibration Period with Fraction A ('Cooling Period')

Refer to Table 3.4 for a detailed description of the experimental numbers and the specific combination of cooling and glycerol equilibration periods used in Experiment 2.

The period between the additions of the two Fractions was termed the 'Cooling Period' for these experiments. After Fraction A was added to all 5 tubes the semen-Fraction A mixture was placed inside the container. The Falcon tube containing Fraction B, a sterile syringe and needle was also placed into the insulating container. This ensured that all equipment to be used would equilibrate to the

changing temperature at the same rate (Bearden *et al.* 2004). The container with its contents was left to equilibrate at room temperature for 30 minutes.

After 30 minutes at room temperature the container containing the 5 tubes as well as Fraction B and the syringe was closed and put into a walk-in cooler (+5°C), to equilibrate further. The rate of cooling from room temperature to refrigerator temperature was -0.25°C/min. It took approximately 2 h for the semen to reach the refrigerator temperature. This rate was calculated by measuring the temperature within the container at 10 minute intervals, until it reached +5°C. These recorded values were then used to calculate the cooling rate.

- 4h after Fraction A was added, the calculated volume of Fraction B was added to Tube E.
- 5h after Fraction A was added, the calculated volume of Fraction B was added to Tube F.
- 6h after Fraction A was added, Fraction B was added to Tube G.
- 7h after equilibration started, Fraction B was added to Tube H.
- 8h after equilibration started, Fraction B was added to Tube I.

3.6.4.2. Semen straws used and information recorded

While waiting to add Fraction B to the different tubes, semen straws were printed for identification. For these experiments French Mini straws (0.25cc) produced by Minitüb (Minitüb Abfüll- und Labortechnik GmbH & Co. KG, Tiefenbach, Germany), were used. A different colour was used for each bull. Information recorded on each straw included: Experiment Date, Bull Identification Number and specific Experiment Number. A Minitüb EasyCoder Straw Printer (Minitüb Abfüll- und Labortechnik GmbH & Co. KG, Tiefenbach, Germany) was used to print the information on the individual straws. Marked straws were also placed in a refrigerator and allowed to equilibrate to +5°C – in order to prevent any temperature fluctuation when the semen was drawn into the straws. This fluctuation in temperature could have had a negative effect on the viability of the spermatozoa (Bearden *et al.* 2004). Equipment to be used for freezing was also set up during this period (See Appendix A, Figure 2.16a and Figure 2.16b). These preparations included logging into the IceCube® computer programme (Minitüb Abfüll- und Labortechnik GmbH & Co.

KG, Tiefenbach, Germany), and allowing the freezing chamber to stabilize at 5°C. A polystyrene container was also filled with liquid nitrogen, which was used to dump straws into the liquid nitrogen, after the semen straws reached -120°C, with the freezing programme.

3.6.4.3. Equilibration Period with Fraction B ('Glycerol Equilibration Period')

Refer to Table 3.4 for a detailed description of experiment numbers and the specific combination of cooling and glycerol equilibration periods used in Experiment 2. Also refer to Figure 3.3 for the standard freezing graph used in the freezing process. The period from Fraction B addition to the onset of the freezing process was termed 'Glycerol Equilibration Period' for these experiments.

3.6.4.3.1. Cooling Period E (4 h)

- 30 minutes after Fraction B was added to Tube E, 5 straws – known as **Treatment E1** – were filled from this tube sealed and frozen using the IceCube® software programme and equipment. When the freezing curve reached -120°C, the straws were removed and immediately plunged into a polystyrene container filled with liquid nitrogen. These straws were then stored in liquid nitrogen, until such time that the straws were thawed for post-freeze evaluation.

- 60 minutes after Fraction B was added to Tube E, 5 straws – known as **Treatment E2** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.

- 120 minutes after Fraction B was added to Tube E, 5 straws – known as **Treatment E3** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.

- 240 minutes after Fraction B was added to Tube E, 5 straws – known as **Treatment E4** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.

3.6.4.3.2. Cooling Period F (5 h)

- 30 minutes after Fraction B was added to Tube F, 5 straws – known as **Treatment F1** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.
- 60 minutes after Fraction B was added to Tube F, 5 straws – known as **Treatment F2** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.
- 120 minutes after Fraction B was added to Tube F, 5 straws – known as **Treatment F3** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.
- 240 minutes after Fraction B was added to Tube F, 5 straws – known as **Treatment F4** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.

3.6.4.3.3. Cooling Period G (6 h)

- 30 minutes after Fraction B was added to Tube G, 5 straws – known as **Treatment G1** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.
- 60 minutes after Fraction B was added to Tube G, 5 straws – known as **Treatment G2** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.
- 120 minutes after Fraction B was added to Tube G, 5 straws – known as **Treatment G3** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.
- 240 minutes after Fraction B was added to Tube G, 5 straws – known as **Treatment G4** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.

3.6.4.3.4. Cooling Period H (7 h)

- 30 minutes after Fraction B was added to Tube H, 5 straws – known as **Treatment H1** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.
- 60 minutes after Fraction B was added to Tube H, 5 straws – known as **Treatment H2** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.
- 120 minutes after Fraction B was added to Tube H, 5 straws – known as **Treatment H3** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.
- 240 minutes after Fraction B was added to Tube H, 5 straws – known as **Treatment H4** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.

3.6.4.3.5. Cooling Period I (8 h)

- 30 minutes after Fraction B was added to Tube I, 5 straws – known as **Treatment I1** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.
- 60 minutes after Fraction B was added to Tube I, 5 straws – known as **Treatment I2** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.
- 120 minutes after Fraction B was added to Tube I, 5 straws – known as **Treatment I3** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.
- 240 minutes after Fraction B was added to Tube I, 5 straws – known as **Treatment I4** – were filled from this tube, sealed and frozen. The same freezing procedure as in Treatment E1 was implemented.

3.6.4.4. Sealing of semen straws for cryopreservation

All straws were sealed using a coloured PVC sealing powder which transforms into a gel-like substance when coming into contact with any liquid – such as the diluted semen. After the semen was drawn into the straws, the open end of each straw was dipped in the powder, which then allowed the powder to come into contact with the semen.

TABLE 3.4: Different experimental numbers used in Experiment 2 with the corresponding cooling time and glycerol equilibration time for each number.

	Experiment Number	Cooling Time (hrs)	Glycerol Equilibration Time (hrs)	Total Equilibration Time (hrs)
EXPERIMENT 2	E1	4	½	4½
	E2	4	1	5
	E3	4	2	6
	E4	4	4	8
	F1	5	½	5½
	F2	5	1	6
	F3	5	2	7
	F4	5	4	9
	G1	6	½	6½
	G2	6	1	7
	G3	6	2	8
	G4	6	4	10
	H1	7	½	7½
	H2	7	1	8
	H3	7	2	9
	H4	7	4	11
	I1	8	½	8½
	I2	8	1	9
	I3	8	2	10
	I4	8	4	12

3.7. Semen storage and thawing procedures

Frozen semen straws were stored in commercial liquid nitrogen (LN_2) flasks. The flasks were refilled with nitrogen once a week.
Straws were thawed individually by placing a straw in a water bath at 35°C, for 10 seconds (See Appendix A, Figure 2.17). This meant that the semen was thawed at a thawing rate of 23.3°C/s. Using clean scissors, the straw was severed in the middle – to obtain a representative semen sample.

3.8. Post-thaw semen evaluation

For post-thawing evaluation, a 75 µl drop of semen was deposited on a warmed sterile microscope slide, and covered with a cover slide (22x22mm). The magnification setting on the microscope was changed to 40x10, in order to observe individual spermatozoal motility. Evaluation of individual motility was performed in several areas of the slide and graded on a scale of 0-100%, with 0% indicating the absence of any linear progressive spermatozoa and 100% that all spermatozoa exhibited linear progressiveness (Mitchell & Doak, 2004). All post-thaw evaluation was done subjectively by the same laboratory technician (See Appendix A, Figure 2.18). The recovery fraction of the semen samples was also determined by using the following formula (Hill *et al.,* 1959):

Recovery Fraction = % Post-Thaw Motility / % Initial Motility

3.9. Statistics

Data were analyzed statistically by means of the GLM model (Statistical Analysis Systems, 2009) for the average effects, over different cooling and glycerol equilibration periods. Repeated Measures Analysis of Variance with the GLM model was used for repeated post-thaw motility rates and recovery fraction evaluations. Means and standard deviations were calculated and significance of difference ($p<0.05$) between means was determined using the Fishers test (Samuels, 1989). Differences with a p-value of $p<0.05$ were considered to differ significantly.

The linear model used, is described by the following equation: $Y = u + T_i + e$ with Y = variable studied during the period (either average post-thaw motility percentage or recovery fractions); u = overall mean of the population; T = effect of the i^{th} treatment of breed; e = error associated with each Y.

CHAPTER 4

RESULTS AND DISCUSSION OF EXPERIMENT 1

4.1. Effect of equilibration period on the mean post-thaw bovine sperm motility

The mean motility are summarized in Table 4.1. Semen samples that were equilibrated for the shortest total equilibration period (4.5 h) yielded a mean post-thaw semen motility rate of 22.5±15.1%. Increasing the total equilibration period of the semen samples to either 5 or 5.5 h resulted in mean post-thaw motility rates of 21.5±13.7 and 23.1±14.6% respectively. The mean post-thaw semen motility rates, resulting from total equilibration periods of 4.5, 5 and 5.5 h respectively were not significantly different from one another.

When applying different equilibration periods of 6, 6.5, 7, 7.5, 8, 8.5 or 9 h to semen samples, the resulting mean post-thaw motility rates recorded were 32.7±12.0, 30.9±13.1, 35.1±11.1, 30.5±13.31, 38.9±9.2, 36.6±11.6 and 38.6±9.6, for the respective periods. The resulting mean post-thaw motility after both 4.5 and 5 h of equilibration (Table 4.1) differed significantly ($p<0.05$) from all the other equilibration periods except for the 5.5 h equilibration. The 5.5 h period yielded a mean post-thaw sperm motility (%) that was significantly lower ($p<0.05$) than the means recorded after periods of 6, 7, 8, 8.5 or 9 h, and also tended to differ from the mean post-thaw motility rates yielded by equilibration periods of 6.5 and 7.5 h. Total equilibration periods of 6, 6.5 and 7.5 all yielded a mean post-thaw sperm motility that was significantly lower ($p<0.05$) than the mean post-thaw motility rates after periods of either 8 or 9 h. The mean post-thaw motility rates resulting from 7, 8 or 9 h of equilibration were not significantly different from one another, although the post-thaw motility rates after 7 h of equilibration was slightly lower than that after 8 or 9 h.

Equilibrating semen samples for longer periods (10, 11 or 12 h) resulted in higher ($p<0.05$) post-thaw motility rates, compared to the shorter equilibration periods (4.5, 5, 5.5, 6, 6.5 or 7.5 h). Periods of 10, 11 and 12 h then yielded post-thaw sperm motility rates of 40.3±7.9%, 41.8±6.0% and 41.2±8.6% respectively. These mean

post-thaw motility rates were not significantly different from one another, or for the post-thaw sperm motility rates subsequent to 8 or 9 h of equilibration. However values were all significantly higher ($p<0.05$) than the mean post-thaw motility after total equilibration periods of 4.5, 5, 5.5, 6, 6.5 and 7.5 h. The mean post-thaw motility resulting from the longer equilibration periods (10, 11 and 12 h) also tended to differ from the post-thaw sperm motility after 7 h of equilibration.

The present results suggest a direct relationship exists between the post-thaw sperm motility and the total equilibration period applied. An increasing equilibration period led to a general linear increase in post-thaw motility rate. However this relationship is only applicable to the equilibration periods applied in this particular study. This relationship between the equilibration period and post-thaw sperm motility is illustrated in Figure 5.1.

Figure 4.1: The relationship between total equilibration period and mean post-thaw motility rate of cryopreserved bovine semen

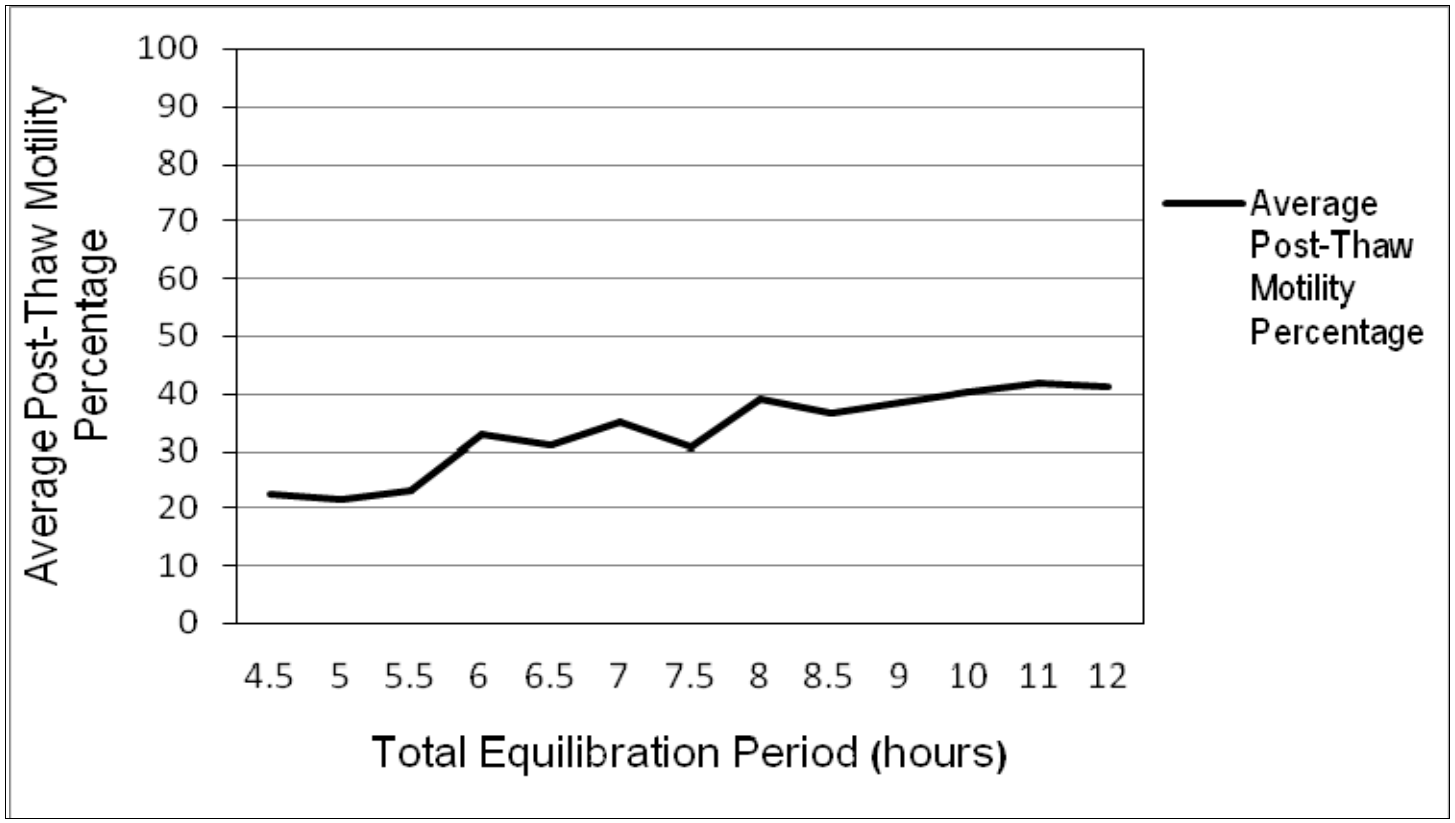


TABLE 4.1: Effects of different total equilibration periods on the mean (±SD) post-thaw motility of bovine spermatozoa

Total Equilibration Time (h)	Mean Post-Thaw Motility Rate (%)
4.5	$22.5^{a} \pm 15.1$
5	$21.5^{a} \pm 13.7$
5.5	$23.1^{abA} \pm 14.6$
6	$32.7^{c} \pm 12.0$
6.5	$30.9^{bBc} \pm 13.1$
7	$35.1^{cdA} \pm 11.1$
7.5	$30.5^{bBc} \pm 13.3$
8	$38.9^{d} \pm 9.2$
8.5	$36.6^{cd} \pm 11.6$
9	$38.6^{d} \pm 9.6$
10	$40.3^{dB} \pm 7.9$
11	$41.8^{dB} \pm 6.0$
12	$41.2^{dB} \pm 8.6$

^{a,b,c} Means in a row with different superscripts differ significantly($p<0.05$)
$^{A.B}$ Means in a row with different superscripts differ significantly ($p<0.1$)

4.2. Effects of equilibration period on the mean recovery fractions of bovine semen

The recovery fractions (% post-thaw sperm motility / % initial sperm motility) are set out in Table 4.2. A higher recovery fraction is the result of a more efficient cryopreservation process. The general trend for the effect of the total equilibration period on the mean recovery fraction of semen is also illustrated in Figure 4.2. The author could not find any previous studies using mean recovery fraction of bovine semen as a variable.

Equilibrating semen samples for a shorter equilibration period (4.5, 5 or 5.5 h) yielded mean recovery fractions of 0.27±0.17, 0.25±0.16 and 0.27±0.17. The mean recovery fractions resulting from equilibration periods of 4.5, 5 or 5.5 h were not significantly different from one another. These recovery fractions did however differ significantly ($p<0.05$) from the recovery fractions yielded by all of the longer equilibration periods (6, 6.5, 7, 7.5, 8, 8.5, 9, 10, 11 or 12 h).

When semen samples were subjected to total equilibration periods of 6, 6.5, 7 or 7.5 h, the resulting mean recovery fractions were 0.39±0.13, 0.36±0.15, 0.41±0.12 and 0.36±0.15 for the respective periods. Total equilibration periods of 6, 6.5 or 7.5 h all yielded mean recovery fractions that were significantly lower ($p<0.05$) than the mean recovery fractions following periods of 8, 8.5, 9, 10, 11 or 12 h. The recovery fraction resulting from 7 h of equilibration differed significantly ($p<0.05$) from the mean recovery fractions resulting from either an 8.5 or 11 h period and also tended to differ from the mean recovery fractions yielded by a 10 or a 12 h equilibration period.

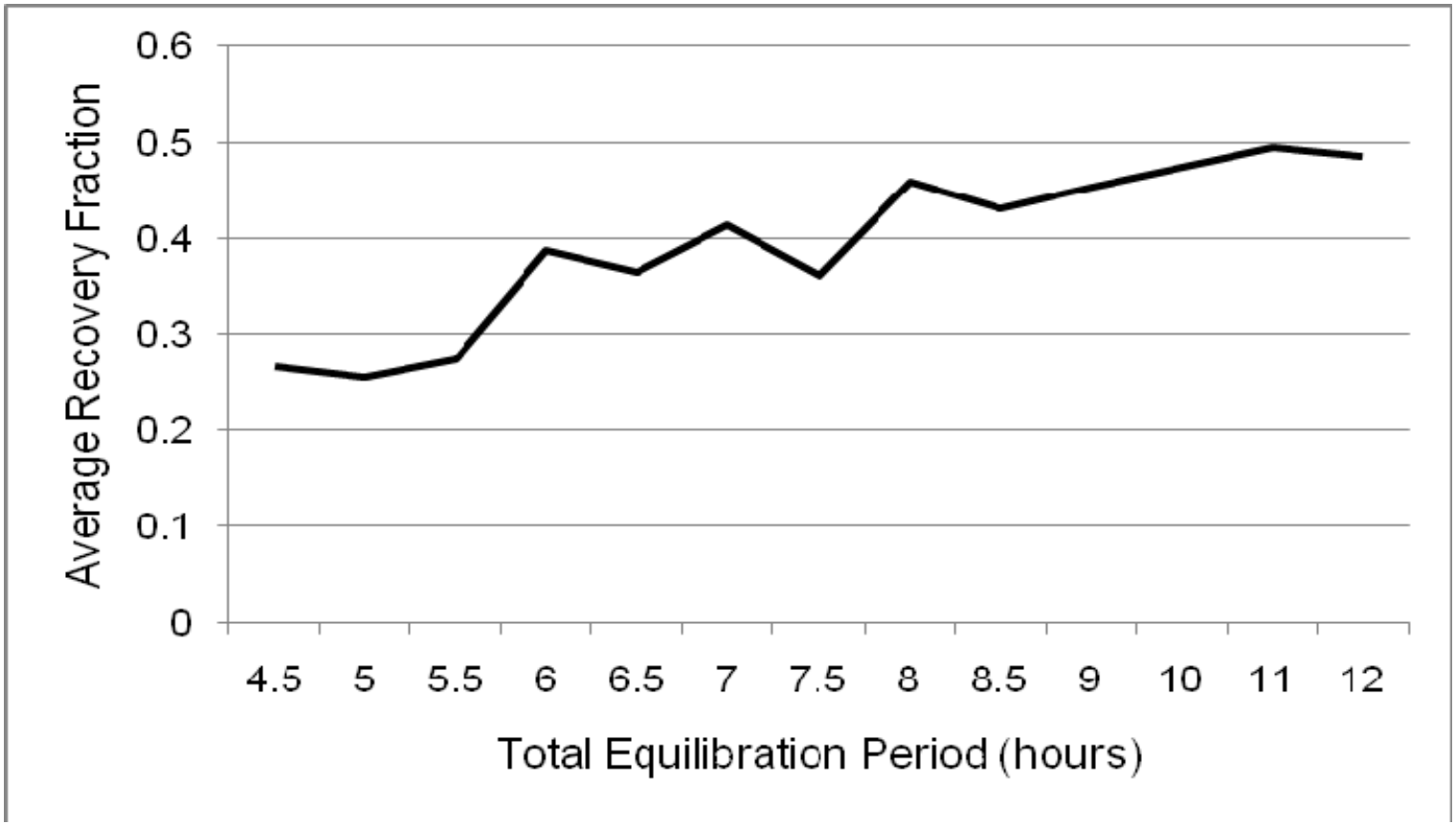
Equilibrating semen samples for longer periods (8, 8.5, 9, 10, 11 or 12 h), resulted in higher ($p<0.05$) recovery fractions, compared to the mean recovery fractions yielded by the shorter equilibration periods (4.5, 5 and 5.5 h). These periods then yielded mean recovery fractions of 0.46±0.10, 0.43±0.13, 0.45±0.10, 0.47±0.08, 0.50±0.06 and 0.49±0.10 respectively. The above-mentioned recovery fractions were however not significantly different from one another.

TABLE 4.2: Effects of different total equilibration periods on the mean (±SD) recovery fractions of bovine spermatozoa

Total Equilibration Time (h)	Mean Recovery Fraction
4.5	$0.27^{a} \pm 0.17$
5	$0.25^{a} \pm 0.16$
5.5	$0.27^{a} \pm 0.17$
6	$0.39^{b} \pm 0.13$
6.5	$0.36^{b} \pm 0.15$
7	$0.41^{bcA} \pm 0.12$
7.5	$0.36^{b} \pm 0.15$
8	$0.46^{cd} \pm 0.10$
8.5	$0.43^{bd} \pm 0.13$
9	$0.45^{cd} \pm 0.10$
10	$0.47^{cBd} \pm 0.08$
11	$0.50^{d} \pm 0.06$
12	$0.49^{cBd} \pm 0.10$

^{a,b,c} Means in a row with different superscripts differ significantly ($p<0.05$)
$^{A.B}$ Means in a row with different superscripts are not significantly different ($p<0.1$)

Figure 4.2: The relationship between the total equilibration period and mean recovery fractions of cryopreserved bovine semen



4.3. Effects of equilibration periods on the mean post-thaw motility rates of semen of Boran and Nguni bulls

The effect of equilibration periods on post-thaw motility are summarized in Table 4.3. The general trend of the effect of equilibration times on the average post-thaw sperm motility in Boran and Nguni semen is illustrated in Figure 4.3. The author could not find any previous studies comparing frozen-thawed sperm motility in Boran and Nguni bulls.

4.3.1. Effects of equilibration time on the post-thaw sperm motility in Boran semen

Semen samples from Boran bulls equilibrated for the shortest equilibration period (4.5 h) recorded a mean post-thaw sperm motility percentage of 17.8±13.3%. By increasing the total equilibration period of the semen samples to either 5 or 5.5 h a slightly higher, though not significantly different, mean post-thaw motility rate of 20.2±15.1 and 20.7±15.6% respectively was recorded. The post-thaw motility rates following equilibration periods of 4.5, 5 and 5.5 h, were not significantly different from one another.

When subjecting the semen samples of the Boran bulls to a total equilibration period of 6, 6.5, 7, 7.5, 8, 8.5 or 9 h, the resulting mean post-thaw sperm motility rates were 30.5±10.0%, 28.7±12.8%, 34.7±6.2%, 28.5±14.4%, 35.6%±8.2%, 33.0±11.9% and 35.3±4.6% for the respective periods. The mean post-thaw motility after both 5 and 5.5 h of equilibration differed significantly ($p<0.05$) from the mean post-thaw motility following equilibration periods of 7, 7.7, 8, 8.5, 9, 10, 11 and 12 h respectively and also tended to be significantly lower than the mean post-thaw sperm motility for the 6 h period. The 4.5 h total equilibration period yielded a mean post-thaw motility rate that was significantly lower ($p<0.05$) than the mean after periods of 6, 7, 8, 8.5, 9, 10, 11 and 12 h, and also tended to differ from the mean post-thaw motility rates yielded by equilibration periods of 6.5 and 7.5 h. The mean post-thaw motility rates recorded from 6, 7, 8 or 9 h of equilibration, were not significantly different from one another.

Equilibrating semen samples from Boran bulls for the longer periods (10, 11 or 12 h) resulted in a mean post-thaw motility rate of 36.6±3.3%, 39.2±3.4% and 38.5±5.8% respectively. These post-thaw motility rates were not significantly different from one

another, or from the mean post-thaw motility rates yielded by 6, 7, 8 or 9 h of equilibration. However the values were all significantly higher ($p<0.05$) than the mean post-thaw sperm motility rates after a total equilibration period of 4.5, 5 and 5.5 h. The total equilibration period of 6.5 and 7.5 h both yielded a mean post-thaw motility that tended to be significantly lower than the mean post-thaw motility rate after an equilibration period of 11 h.

4.3.2. The effects of total equilibration period on the post-thaw sperm motility in Nguni bulls

The shortest equilibration periods (4.5, 5 and 5.5 h) applied to semen samples obtained from Nguni bulls yielded a mean post-thaw motility of 27.2±15.8%, 22.8±13.6% and 25.4±14.5%. These mean post-thaw motility rates following a total equilibration period of 4.5, 5 and 5.5 h were not significantly different.

The equilibration of semen samples from the Nguni bulls for a total equilibration period of 6, 6.5, 7 or 7.5 h, resulted in a mean post-thaw motility rate of 34.9±13.2%, 33.1±13.8%, 35.5±13.6% and 32.6±13.1% for the respective periods. The mean post-thaw motility rates after 4.5 h of the total equilibration period tended to differ from the post-thaw motility rates recorded by equilibration periods of 6 and 7 h. The 5 and 5.5 h periods both yielded a mean post-thaw sperm motility that was significantly lower ($p<0.05$) than the post-thaw motility after equilibration periods of 6 and 7 h. The mean post-thaw motility after a 5 h total equilibration period also differed significantly ($p<0.05$) from the mean post-thaw motility rate, yielded after a period of 6.5 h and tended to differ from the post-thaw motility after 7.5 h of equilibraton.

Subjecting semen samples from Nguni bulls to longer total equilibration periods generally resulted in slightly higher ($p<0.05$) post-thaw sperm motility. Periods of 8, 8.5, 9, 10, 11 and 12 h yielded a mean post-thaw motility of 42.3±8.9%, 40.2±11.1%, 41.9±11.1%, 43.9±8.7%, 44.4±6.5% and 43.9±9.7% respectively. All these post-thaw motility rates were not significantly different from one another. However the values were all significantly higher ($p<0.05$) than the motility rates after equilibration periods of 4.5, 5 and 5.5 h. Total equilibration periods of 6, 6.5 and 7.5 h all yielded post-thaw motility rates that were significantly lower ($p<0.05$) than the mean post-thaw

motility rates after periods of 8, 9, 10, 11 or 12 h. Average post-thaw sperm motility after total equilibration periods of 8, 10 and 11 h differed significantly ($p<0.05$) from the mean post-thaw motility after 7 h of equilibration. Total equilibration periods of either 9 or 12 h also resulted in a mean post-thaw motility rate that tended to be higher than the mean post-thaw sperm motility following a 7 h equilibration period.

4.3.3. Effects of total equilibration period and breed on the mean post-thaw sperm motility

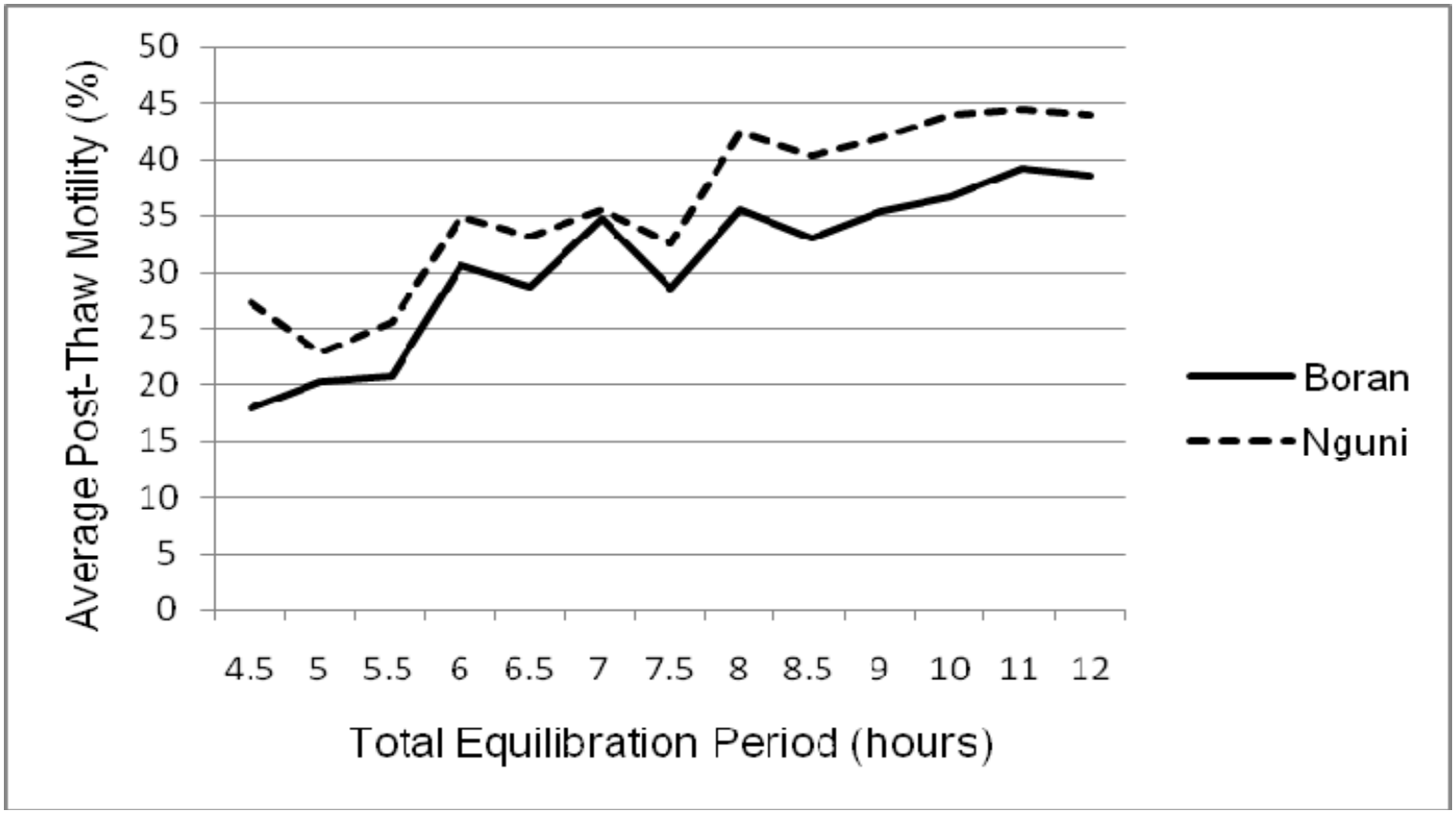
The mean post-thaw motility of semen collected from the Nguni bulls was significantly higher ($p<0.05$) than that of semen collected from Boran bulls, after 8 or 9 h of equilibration. With a total equilibration period of 10 h, the post-thaw sperm motility rate of the Nguni bulls tended to be higher, compared to semen from the Boran bulls. After total equilibration periods of 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8.5, 11 or 12 h the difference between the mean post-thaw motility recorded by the Boran and Nguni bulls were not significantly different.

TABLE 4.3: Effects of different equilibration period on the mean (±SD) post-thaw sperm motility of Boran and Nguni bulls

Total Equilibration Time (h)	BREED	
	Boran Mean Post-Thaw Motility (%)	Nguni Mean Post-Thaw Motility (%)
4.5	17.8^{aA} ± 13.3	27.2^{aAb} ± 15.8
5	20.2^{abA} ± 15.1	22.8^{bA} ± 13.6
5.5	20.7^{abA} ± 15.6	25.4^{bc} ± 14.5
6	30.5^{bBc} ± 10.0	34.9^{aBd} ± 13.2
6.5	28.7^{aBcA} ± 12.8	33.1^{acd} ± 13.8
7	34.7^{c} ± 6.2	35.5^{aBdeA} ± 13.6
7.5	28.5^{aBcA} ± 14.4	32.6^{abBd} ± 13.1
8	$^{a}35.6^{c}$ ± 8.2	$^{b}42.30^{f}$ ± 8.9
8.5	33.0^{c} ± 11.9	40.2^{df} ± 11.1
9	$^{a}35.3^{c}$ ± 4.6	$^{b}41.9^{eBf}$ ± 11.1
10	$^{aA}36.6^{c}$ ± 3.3	$^{aB}43.9^{f}$ ± 8.7
11	39.2^{cB} ± 3.4	44.4^{f} ± 6.5
12	38.5^{c} ± 5.8	43.9^{eBf} ± 9.7

a,b,c Means in the same column with different superscripts differ significantly ($p<0.05$)
a,b,c Means in the same row with different superscripts differ significantly ($p<0.05$)
A.B Means in the same column with different superscript letter tended to differ ($p<0.1$)
A.B Means in the same row with different superscript letter tended to differ ($p<0.1$)

Figure 4.3: The relationship between the total equilibration period and mean post-thaw motility rate of cryopreserved semen from Boran and Nguni bulls



4.4. Effects of equilibration periods on the mean recovery fraction of semen from Boran and Nguni bulls

The effect of equilibration times on the recovery fraction is summarized in Table 4.4 and the general trend on the mean recovery fraction of Boran and Nguni semen is illustrated in Figure 4.4.

The author could not find any previous studies using the mean recovery fraction of bovine semen as a variable and comparing frozen-thawed semen from Boran and Nguni bulls.

4.4.1. Effect of equilibration period on the mean recovery fraction of Boran semen

The shortest equilibration period (4.5 h) applied to semen samples from the Boran bulls recorded an mean recovery fraction (0.22±0.17). Increasing the total equilibration period of the semen samples to either 5 or 5.5 h resulted in slightly increasing the value – although not significantly significant (mean recovery fractions of 0.247±0.185 and 0.252±0.190, respectively). The mean recovery fractions following total equilibration periods of 4.5, 5 and 5.5 h were not significantly different from one another.

When semen samples from the Boran bulls were allowed to equilibrate for periods of 6, 6.5, 7, 7.5, 8 or 8.5 h the resulting recovery fractions were 0.37±0.13, 0.35±0.16, 0.42±0.08, 0.35±0.18, 0.43±0.10, 0.40±0.15 for the respective equilibration periods. The recorded recovery fraction after 4.5, 5 and 5.5 h of equilibration differed significantly ($p<0.05$) from that of the equilibration periods for 6, 7, 8, 8.5 and 12 h. The recovery fraction following 4.5 h of equilibration tended to be lower than the mean recovery fractions following 6.5 and 7.5 h periods. The mean recovery fraction resulting from 6, 6.5, 7, 7.5, 8 or 8.5 h of equilibration was not significantly different.

Equilibrating semen samples from Boran bulls for longer periods (9, 10, 11 or 12 h) resulted in the highest ($p<0.05$) recovery fractions being recorded. Periods of 9, 10, 11 and 12 h yielded mean recovery fractions of 0.42±0.06, 0.44±0.05, 0.47±0.05 and 0.46±0.08 respectively. These recovery fractions were not significantly different. The mean recovery fractions after equilibration periods of 4.5, 5 or 5.5 h again were

significantly lower ($p<0.05$) than the recovery fractions yielded following equilibration periods of 9, 10, 11 or 12 h. Total equilibration periods of 6, 6.5 and 7.5 h both yielded a mean recovery fraction that tended to be significantly lower ($p<0.1$) than the mean recovery fraction after a period of 11 h. The mean recovery fraction after 6.5 and 7.5 h of equilibration was also significantly lower ($p<0.05$) than the recovery fraction recorded after 12 h of equilibration.

4.4.2. Effects of equilibration period on the mean recovery fraction of Nguni semen

The shortest equilibration period (4.5 h) applied to semen samples from the Nguni bulls recorded an mean recovery fraction of 0.31±0.18. By increasing the total equilibration period of the semen samples to either 5 or 5.5 h, resulted in slightly lower recovery fractions of 0.26±0.15 and 0.29±0.16, respectively. The means resulting from the equilibration periods of 4.5, 5 and 5.5 h were not significantly different.

When applying total equilibration periods of 6, 6.5, 7, 7.5, 8, 8.5 or 9 h to several semen samples from Nguni bulls, the resulting mean recovery fractions were 0.40±0.14, 0.38±0.15, 0.41±0.15, 0.38±0.14, 0.49±0.09, 0.46±0.11 and 0.48±0.12 for the respective periods. The recovery fractions after 4.5, 5.5 or 5 h of equilibration all differed significantly ($p<0.05$) from the equilibration periods of 8, 8.5 and 9 h. The 4.5 h period also yielded a mean recovery fraction that tended to be lower from the mean recovery fractions after periods of 6 and 7 h. The mean recovery fraction resulting from a 5 h equilibration period was significantly lower ($p<0.05$) than recovery fractions following equilibration periods of 6, 6.5, 7 or 7.5 h. A total equilibration period of 5.5 h yielded a mean recovery fraction significantly lower ($p<0.05$) than the mean recovery fraction after either 6 or 7 h. The recovery fractions recorded for 6, 6.5, 7 or 7.5 h of equilibration differed significantly ($p<0.05$) from the mean recovery fractions after either 8 or 9 h. Total equilibration periods of 8, 8.5 and 9 h resulted in recovery fractions that were not significantly different.

Equilibrating semen samples from Nguni bulls for longer periods of time (10, 11 or 12 h) resulted in higher ($p<0.05$) recovery fractions. Periods of 10, 11 and 12 h yielded mean recovery fractions of 0.51±0.08, 0.52±0.07 and 0.51±0.11, respectively. These

mean recovery fractions were not significantly different. The mean recovery fraction resulting from the shorter equilibration periods of 4.5, 5, 5.5, 6, 6.5, 7 and 7.5 h were all significantly lower ($p<0.05$), than the mean fractions recorded by longer equilibration periods of 10, 11 and 12 h.

4.4.3. Effects of equilibration periods and breed on mean recovery fractions of semen

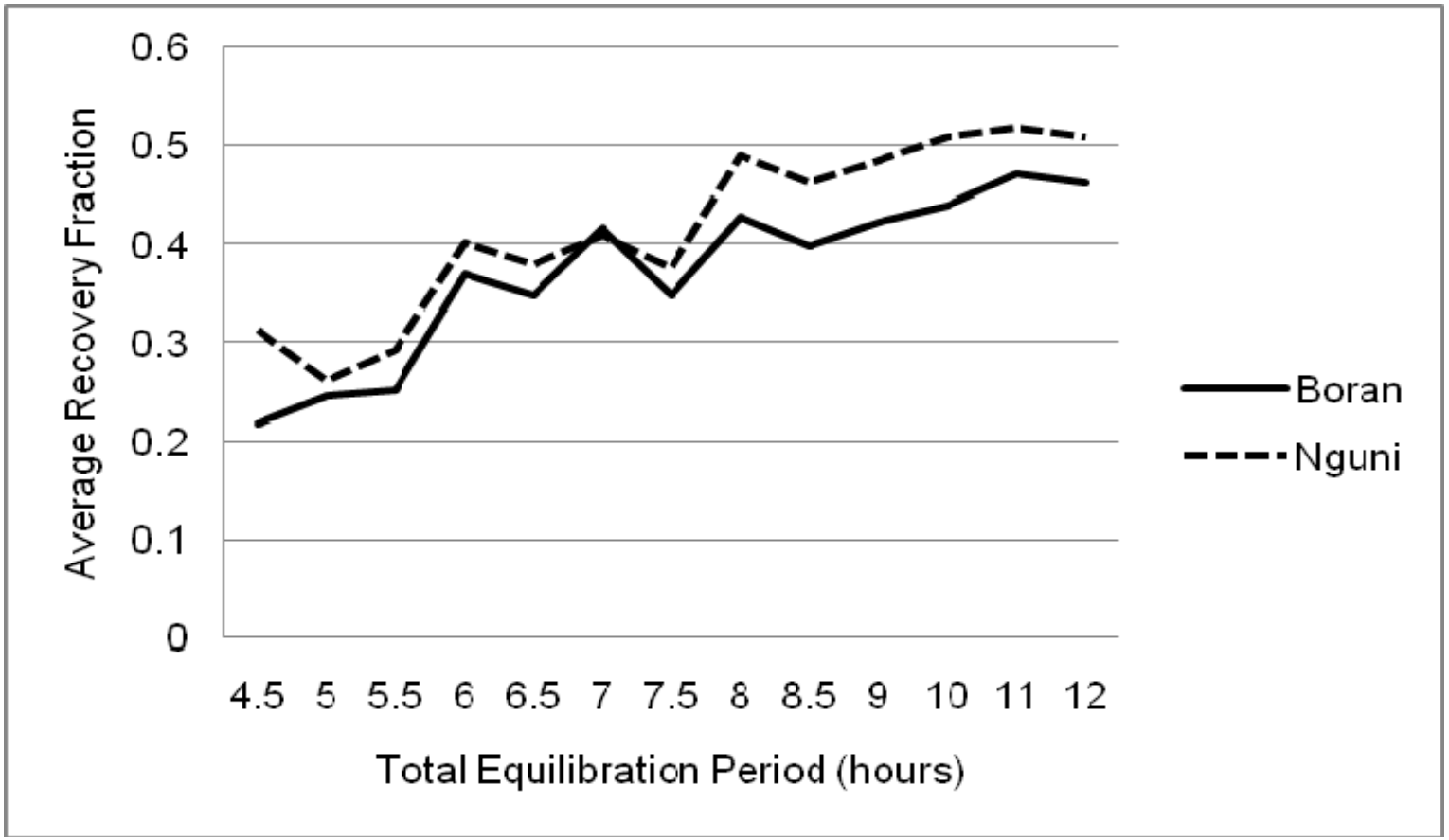
The mean recovery fractions of semen collected from the Nguni and Boran bulls after either 8 or 9 h of equilibration suggest that the Nguni bulls tended to have higher recovery fractions. After total equilibration periods of 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8.5, 10, 11 or 12 h the difference between the mean recovery fractions yielded by the Boran and Nguni bulls were not significantly different.

TABLE 4.4: Effects of different equilibration periods on the mean (±SD) recovery fraction of Boran and Nguni spermatozoa

Total Equilibration Time (h)	BREED	
	Boran	**Nguni**
	Mean Recovery Fraction	Mean Recovery Fraction
4.5	$0.218^{aA} \pm 0.17$	$0.313^{aAb} \pm 0.18$
5	$0.247^{a} \pm 0.19$	$0.261^{b} \pm 0.15$
5.5	$0.252^{a} \pm 0.19$	$0.293^{bc} \pm 0.16$
6	$0.371^{bA} \pm 0.13$	$0.402^{aBd} \pm 0.14$
6.5	$0.348^{aBbAcA} \pm 0.16$	$0.379^{acd} \pm 0.15$
7	$0.418^{b} \pm 0.08$	$0.409^{aBd} \pm 0.15$
7.5	$0.347^{aBbAcA} \pm 0.18$	$0.376^{acd} \pm 0.14$
8	$^{\underline{aA}}0.429^{b} \pm 0.10$	$^{\underline{aB}}0.490^{e} \pm 0.09$
8.5	$0.398^{b} \pm 0.15$	$0.462^{de} \pm 0.11$
9	$^{\underline{aA}}0.423^{b} \pm 0.06$	$^{\underline{aB}}0.484^{e} \pm 0.12$
10	$0.440^{b} \pm 0.05$	$0.508^{e} \pm 0.08$
11	$0.472^{bB} \pm 0.05$	$0.518^{e} \pm 0.07$
12	$0.463^{bcB} \pm 0.08$	$0.508^{e} \pm 0.11$

^{a,b,c} Means in the same column with different superscripts differ significantly ($p<0.05$)
$^{\underline{a.b.c}}$ Means in the same row with different superscripts differ significantly ($p<0.05$)
$^{A.B}$ Means in the same column with different superscript letter tended to differ ($p<0.1$)
$^{\underline{A.B}}$ Means in the same row with different superscript letter tended to differ ($p<0.1$)

Figure 4.4: The relationship between the equilibration period and mean recovery fraction of cryopreserved semen from Boran and Nguni bulls



4.5. Effects of different cooling and glycerol equilibration periods on the post-thaw sperm motility of bovine semen

The effect of cooling and glycerol equilibration periods on the post-thaw sperm motility is set out in Table 4.5.Further the general trends for cooling on the post-thaw motility of Boran and Nguni semen is illustrated in Figure 4.5. The trend for the effect of the different glycerol equilibration periods on post-thaw motility is also illustrated in Figure` 4.6.

4.5.1. Effects of different cooling periods on the post-thaw sperm motility in bovine semen

Overall, a cooling period of 240 minutes recorded the best (not significantly different) results, with an overall mean post-thaw motility of 40.7±7.8%. A cooling period of 120 minutes yielded the second highest (not significantly different) post-thaw motility of 37.6±9.4%. Cooling periods of 30 (28.7±14.3%) and 60 minutes (32.4±13.5%) resulted in the lowest ($p<0.05$) and second lowest ($p<0.05$) post-thaw sperm motility, respectively. The mean post-thaw motility after a cooling period of 30 and 60 minutes both differed significantly ($p<0.05$) from each other, as well as the mean post-thaw sperm motility recorded from the other cooling periods. The mean post-thaw motility after 120 and 240 minutes of cooling, tended to be different.

Dhami and Sahni (1993) found that a 2 h cooling period recorded a significantly higher ($p<0.01$) post-thaw motility, than a 1 h cooling period. In the present study a similar tendency was observed. Smith and Merilan (1991) determined that a slow cooling rate (cooling semen to 5°C over a 3 h period (34.5%), compared to a faster cooling rate – where semen was cooled to 5°C, within 0.5 h (31.5%)) was beneficial for the post-thaw sperm survival of Holstein bulls. Griffin (2004) again reported a cooling period of 4 h to be superior to a 2 h cooling period for the spermatozoa of Brangus bulls. Ennen *et al.* (1976) reported a cooling period of either 120 or 240 minutes to be superior to a cooling period of only 30 minutes. This is in agreement with the results of the present study, although Ennen *et al.* (1976) used semen collected from Angus and Hereford bulls.

When comparing the different cooling periods following 4 h of glycerol equilibration, the post-thaw motility after 30 minutes (22.5±15.1%) was significantly lower

($p<0.05$) than the mean post-thaw sperm motility after either 120 (32.1±11.5%) minutes or 240 (39.1±9.1%) minutes. A 60 minute cooling period also resulted in a significantly lower post-thaw motility ($p<0.05$) (21.5±13.7%) when compared to both 120 minutes and 240 minutes. There was also a tendency towards a significant difference between the mean post-thaw sperm motility of 120 and 240 minutes. A cooling period of 30 minutes resulted in a post-thaw motility percentage slightly lower than that after 60 minutes, but no significant difference between the two post-thaw motility rates were recorded.

With 5 h of glycerol equilibration significant differences ($p<0.05$) between the post-thaw sperm motility were recorded for cooling times following 30 (23.1±14.6%) and 60 (33.3±12.9%) minutes, 30 and 120 (35.8±9.0%) minutes, as well as 30 and 240 (40.0±8.82) minutes – with 30 minutes resulting in a lower mean post-thaw sperm motility, compared to the other periods. Although a cooling period of 240 minutes gave the highest mean post-thaw motility, it was not significantly higher than either 120 or 60 minutes. The 120 minute cooling period resulted in the second highest mean post-thaw motility, while a cooling period of 60 minutes recorded the second lowest value.

With 6 h of glycerol equilibration, a cooling period of 30 minutes yielded a significantly lower ($p<0.05$) mean post-thaw sperm motility rate (30.9±13.1%), than either a 120 (41.1±7.0%) or 240 (41.1±7.0%) minutes. A cooling period of 60 minutes yielded an mean post-thaw motility rate (34.4±13.2%) that was higher (not significant different) than the mean post-thaw motility rate after 30 minutes. However still lower (no significant difference) than the mean post-thaw motility after 120 and 240 minutes. There were no significant differences between the mean post-thaw motility rates resulting from cooling periods of 60, 120 and 240 minutes, as well as between the mean post-thaw motility when comparing cooling periods of 30 and 60 minutes.

With 7 h of glycerol equilibration, 30 minutes of cooling resulted in the lowest (differed significantly from 120 and 240 minutes; $p<0.05$) mean post-thaw motility (30.5±13.3%) while a 60 minute cooling period yielded the second lowest (no significant difference) post-thaw motility (36.5±10.9%) and 240 minutes cooling recorded the highest (no significant difference) post-thaw motility (41.8±6.0%). The

mean post-thaw sperm motility after 30 and 120 (39.8±7.6%) minutes, as well as after 30 and 240 minutes of cooling recorded significant differences ($p<0.05$). When comparing the post-thaw motility after cooling periods of 30 and 60 minutes, no significant differences were recorded. Comparing post-thaw motility resulting after cooling periods of 60, 120 and 240 minutes, revealed no significant differences.

No significant differences between the mean post-thaw motility following the respective cooling periods were compared, when combined with 8 h of glycerol equilibration. The lowest (not significantly different) mean post-thaw sperm motility (36.0±11.8%) was following 60 minutes of cooling. A 30 minute cooling period yielded a slightly higher post-thaw motility (36.6±11.6%), while the cooling period of 240 minutes gave the highest mean post-thaw sperm motility (41.2±8.6%) and the 120 minute period resulted in the second highest post-thaw motility (39.4±8.8%) (not significantly different).

4.5.2. Effects of different glycerol equilibration periods on the post-thaw motility of bovine semen

When glycerol equilibration periods were compared over all the cooling periods of 4, 5, 6, 7 and 8 h yielded post-thaw motility rates of 28.8±14.3%, 33.0±13.0%, 33.0±13.0%, 37.2±10.5% and 38.3±10.3% respectively. At the $p<0.05$ level, the mean post-thaw motility after 4 h of equilibration differed significantly from all the other equilibration periods. The mean post-thaw motility after 5 h differed significantly ($p<0.05$) from the 7 and 8 h. There was a tendency towards significance between the mean post-thaw motility rates after 5 and 6 h and no significant differences were recorded between the post-thaw motility rates after the glycerol equilibration periods of 6, 7 and 8 h.

Gilbert and Almquist (1978) studied the effects of glycerol equilibration for periods of 0, 3 or 9 h, and found that the 0 h period was significantly lower in terms of motility than either the 3 or 9 h period. The resulting motility after 3 or 9 h of glycerol equilibration did however not differ significantly. Wiggin and Almquist (1974) reported that a 2 h glycerol equilibration period was optimal for semen frozen in straws, and that glycerol equilibration periods exceeding 4 h were not recommended. Tuli *et al.*(1981) determined that a 4 h glycerol equilibration period yielded superior post-

thaw sperm motility than glycerol equilibration periods of either 2 of 6 h. This is not in agreement with the results of the present study, which found a 6 h glycerol equilibration period to be superior to 4 h of glycerol equilibration. Griffin (2004) also found glycerol equilibration periods of either 4 or 6 h to be optimum for freezing bovine semen. Herold e*t al.* (2006) studied the effects of glycerol equilibration periods of 2, 3, 4, 5, 6, 7, 8 and 9 h on the post-thaw motility of African buffalo semen and found no significant differences between the different glycerol equilibration periods. Similarly Graham *et al.* (1956) also found no significant difference ($p<0.05$) between the non-return rates of cows inseminated with semen that were equilibrated with glycerol for either 4 or 8 h. The results of the present study found a significant difference ($p<0.05$) between the mean post-thaw motility rates after glycerol equilibration periods of 4 and 8 h.

Miller and VanDemark compared glycerol equilibration periods of 2, 6 and 18 h and found a 6 h period to be superior to either the 2 or 18 h periods (O'Dell and Hurst, 1955). Saroff and Mixner (1954) studied glycerol equilibration periods of 2, 6, 12 and 18 h and concluded that there was a direct relationship between post-thaw sperm motility and the glycerol equilibration period. Sullivan and Mixner (1963) also researched glycerol equilibration periods of 2, 6, 12 and 18 h and found that a 2 h period was inferior to any of the longer glycerol equilibration periods (6, 12 or 18 h).

A study done by Ennen *et al.* (1976) to compare post-thaw motility after cooling for 240 minutes and glycerol equilibration periods of 1, 2, 4, 8 or 16 h found no significant difference between results after glycerol equilibration periods of 1, 2, 4 or 8 h. It was also found that equilibration periods of 1, 2, 4, 8 and 16 h resulted in mean post-thaw motility rates of 31.2±0.5%, 31.1±0.4%, 30.7±0.4%, 30.2±0.4% and 28.8±0.4% respectively.

Cragle *et al.* (1955) did a series of experiments in order to determine the optimum glycerol equilibration period for bovine spermatozoa, and proposed that 14.9 h of equilibration resulted in the highest motility. Cooling semen for a cooling period of 30 minutes, combined with a glycerol equilibration period of 4 h resulted in a significantly lower ($p<0.05$) mean post-thaw motility (22.5±15.1%), when compared to the mean post-thaw sperm motility rates after the same cooling period, combined with 6 h (30.9±13.1%) and 8 h (36.6±11.6%) of equilibration respectively. There was

also a tendency towards a significant difference between the mean post-thaw motility rates at 4 h and 7 h (30.5±13.3%), with 7 h resulting in a better mean. A glycerol equilibration period of 5 h (23.1±14.6%) yielded an mean post-thaw motility rate that was significantly lower ($p<0.05$) than that at 8 h (36.6±11.6%). It was also slightly different when compared to the higher post-thaw motility after both 6 h (30.9±13.1%) and 7 h (30.5±13.3%). Although the differences between the mean post-thaw motility after 4 and 5 h equilibration were not significantly significant, a 5 h period yielded a slightly higher post-thaw motility. Eight h of equilibration resulted in a higher (not significantly different) mean post-thaw motility rate, than either 6 or 7 h. The mean post-thaw sperm motility after 6 h was higher than after 7 h – although not significantly different.

A cooling period of 60 minutes and 4 h of glycerol equilibration resulted in a mean post-thaw motility (21.5±13.7%), significantly lower ($p<0.05$) than at 5 h (33.3±12.9%), 6 h (34.4±13.2%), 7 h (36.5±10.9%) and 8 h (36.0±11.8%) (although still low) of equilibration. After 60 minutes of cooling and 7 h of equilibration the highest (not significantly different) mean post-thaw motility was recorded, with decreasing motility after 8, 6 and 5 h. The differences between the mean post-thaw motility rates of 5, 6, 7 and 8 h were not significantly significant.

With a cooling period of 120 minutes, there was a significant difference ($p<0.05$) between the mean post-thaw sperm motility rates after 4 h (32.1±11.5%) and 6 h (41.1±7.0%) of glycerol equilibration – with 4 h resulting in an mean 9% lower than after 6 h. When comparing the mean post-thaw sperm motility after 4 and 7 h (39.8±7.6%), as well as 4 and 8 h (39.4±8.8%) they were slightly different. The differences between mean post-thaw motility after equilibration periods of 5, 6, 7 and 8 h were not significantly significant.

With a cooling period of 240 minutes there were no significant differences recorded between the mean post-thaw motility rates of the different glycerol equilibration periods. An equilibration period of 7 h (41.8±6.0%) resulted in the highest (not significant) mean post-thaw motility, followed by 8 h (41.2±8.6%) and 6 h (41.1±7.0%). Five (40.0±8.8%) and 4 h (39.1±9.1%) of glycerol equilibration yielded lower (although not significant), post-thaw motility rates, when compared to the other periods.

TABLE 4.5: The overall effects of different cooling and glycerol equilibration periods on the mean (±SD) post-thaw motility of bovine spermatozoa

GLYCEROL EQUILIBRATION PERIOD (h)	COOLING PERIOD (minutes) 30	60	120	240	Mean
4	22.5^{abdB} ±15.14	21.5^{af} ±13.73	32.1^{klnBoBpB} ±11.47	39.1^{kBp} ±9.13	**28.8^{a} ±14.27**
5	23.1^{abcBdB} ±14.57	33.3^{ghijlq} ±12.94	35.8^{gklmnoq} ±8.96	40.0^{glpq} ±8.82	**33.0^{bA} ±12.98**
6	30.9^{bBcdeh} ±13.11	34.4^{cghijmr} ±13.21	41.1^{hlmnor} ±6.99	41.1^{hmpr} ±7.00	**36.9^{bB,c} ±11.26**
7	30.5^{aBbBcde} ±13.31	36.5^{dghijns} ±10.91	39.8^{ikBlmnos} ±7.60	41.8^{inps} ±5.97	**37.2^{c} ±10.57**
8	36.6^{cdejo} ±11.59	36.0^{eghijot} ±11.75	39.4^{jkBlmnot} ±8.84	41.2^{ejopt} ±8.57	**38.3^{c} ±10.27**
Over-all mean ±SD	**28.7^{a} ±14.25**	**32.4^{b} ±13.46**	**37.6^{cA} ±9.37**	**40.7^{cB} ±7.82**	

a,b,c Means in the same row or column with different superscripts differ significantly (p<0.05)
a,b,c Means in the same row or column with different superscripts differ significantly (p<0.05)
A.B Means in the same row or column with different superscripts tended to differ (p<0.1)
A.B Means in the same row or column with different superscripts tended to differ (p<0.1)

Figure 4.5: The relationship between cooling period and mean post-thaw sperm motility rate of cryopreserved bovine semen

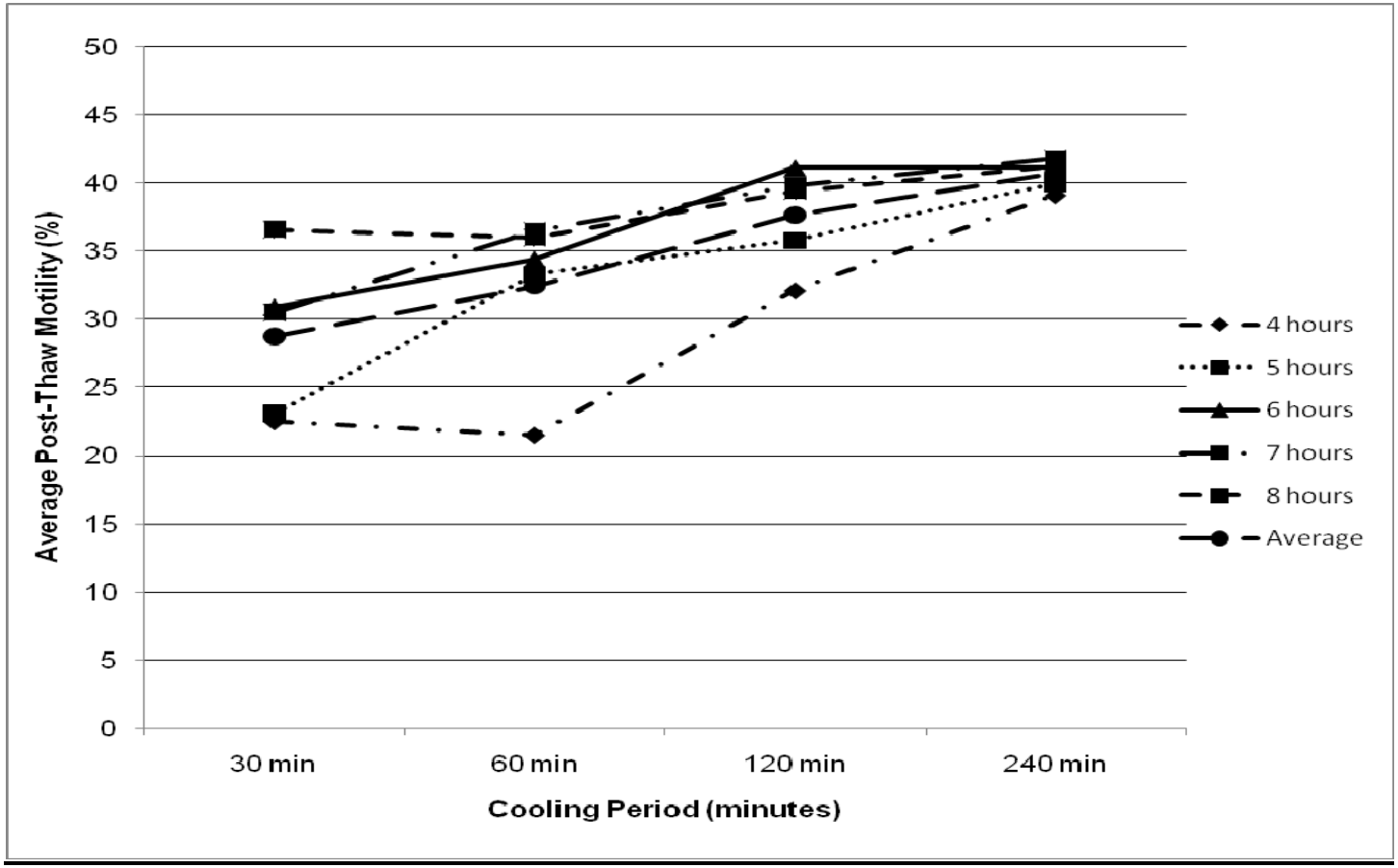
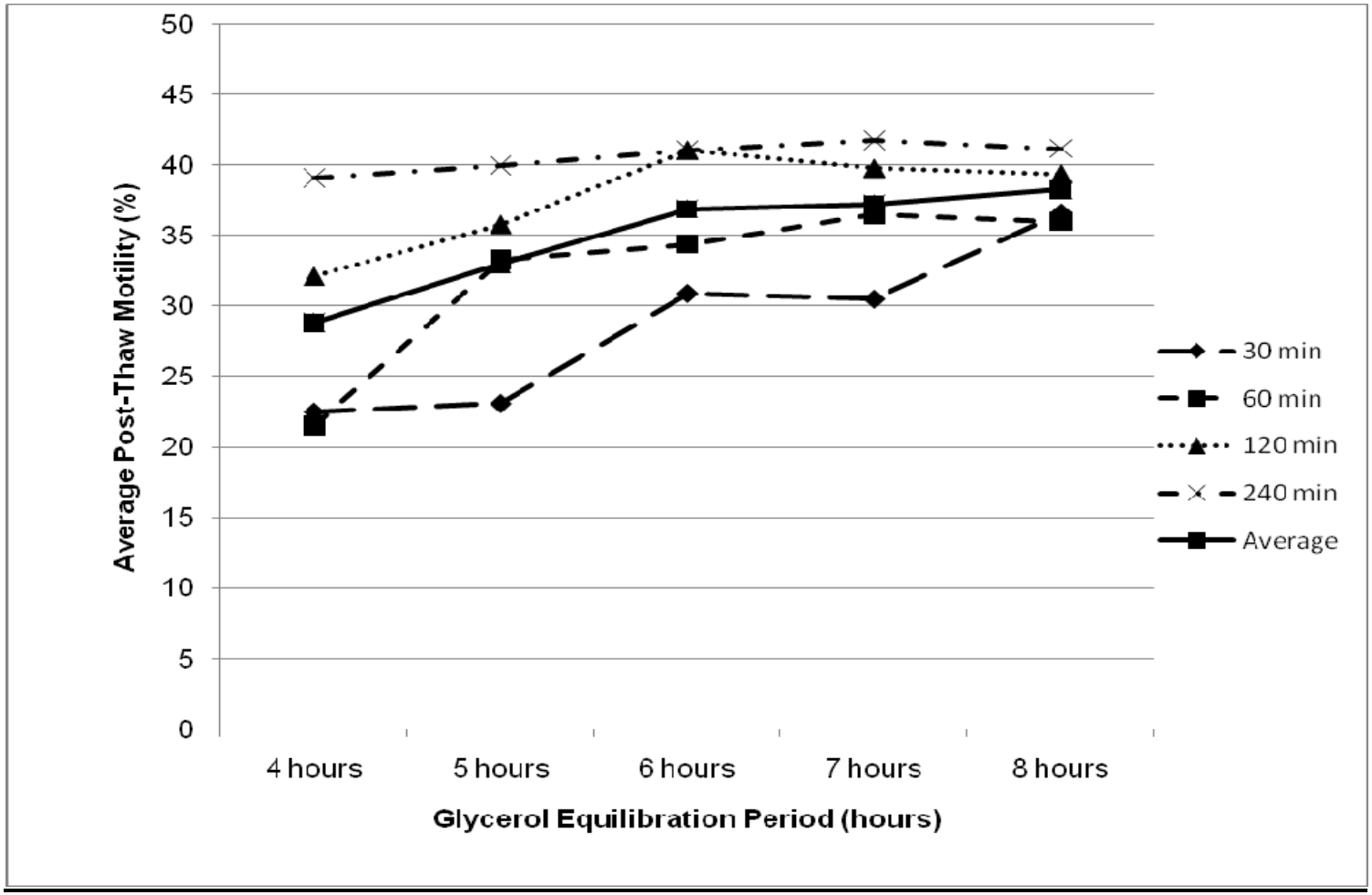


Figure 4.6: The relationship between glycerol equilibration period and mean post-thaw sperm motility rate of cryopreserved bovine semen



4.6. Effects of different cooling and glycerol equilibration periods on the recovery fractions of bovine semen

The effects of the different cooling and glycerol equilibration periods on the recovery fractions are summarized in Table 4.6. The trend for the effect of different cooling periods on the mean recovery fraction of bovine semen is also illustrated in Figure 4.7, together with the effect of different glycerol equilibration periods on the mean recovery fraction of bovine semen (Figure 4.8).

The author could not find any previous studies using mean recovery fraction of bovine semen as a variable for evaluating post-thaw sperm motility..

4.6.1. Effect of different cooling periods on the mean recovery fractions of bovine semen

Over all the glycerol equilibration periods, the mean recovery fraction increased as the period of cooling increased. Mean recovery fractions for cooling periods of 30, 60, 120 and 240 minutes were 0.34±0.16, 0.38±0.15, 0.44±0.10 and 0.48±0.09 respectively. The fraction after 30 minutes was significantly lower ($p<0.05$) than the mean recovery fractions after 60, 120 and 240 minutes. While the mean recovery fraction after 60 minutes was also significantly lower ($p<0.05$), than that after 120 and 240 minutes. The mean fractions after 120 and 240 minutes were not significantly different.

When a glycerol equilibration period of 4 h was combined with several cooling periods, there were significant differences ($p<0.05$) between the mean recovery fraction of semen cooled for 30 (0.27±0.17) and 120 (0.38±0.13) minutes, 30 and 240 (0.46±0.10) minutes, 60 (0.25±0.16) and 120 minutes, as well as 60 and 240 minutes. Values tended towards being significantly different for the recovery fractions of 120 and 240 minutes. A 60 minute cooling period followed by 4 h of glycerol equilibration resulted in the lowest ($p<0.05$) from 120 and 240 minutes. The mean recovery fraction and a cooling period of 30 minutes yielded the second lowest ($p<0.05$) – from 120 and 240 minutes, mean recovery fraction, while the 240 and 120 minutes of cooling resulted in the highest ($p<0.05$, from 30 and 60 minutes) and second highest ($p<0.05$, from 30 and 60 minutes) mean recovery fractions.

With a 5 h period of glycerol equilibration the mean recovery fraction after 30 minutes of cooling (0.27±0.17) differed significantly ($p<0.05$) from the mean recovery fractions – after cooling periods of 60 (0.39±0.14), 120 (0.42±0.10) as well as 240 minutes (0.47±0.10). A tendency to differ was recorded ($p<0.1$), when comparing the mean recovery fractions after 60 and 240 minutes cooling periods. The mean recovery fractions after 120 and 240 minutes were not significantly different. The mean recovery fraction recovered after 30 minutes of cooling and 5 h of equilibration was the lowest ($p<0.05$) and the mean after 240 minutes the highest (differed ($p<0.05$) from 30 minutes, tended to differ from 60 minutes). Cooling for 60 and 120 minutes resulted in the second lowest (not significant from the 240 minute period) and second highest ($p<0.05$ from the 30 minute period) mean fractions, respectively.

When employing a 6 h glycerol equilibration period, the cooling periods of 30 (0.36±0.15) and 120 (0.49±0.08) minutes as well as 30 and 240 (0.48±0.07) minutes recorded significant differences ($p<0.05$) regarding the respective mean recovery fractions. Cooling periods of 60 (0.40±0.15) and 120 minutes, as well as 60 and 240 minutes all resulted in a mean recovery fractions that tended to be different. The mean recovery fractions from 30 and 60 minutes as well as 120 and 240 minutes of cooling were not significantly different. Once again 30 and 60 minutes of cooling yielded the lowest ($p<0.05$) and second lowest (compared to 120 and 240 minutes) mean recovery fractions. Cooling for 120 minutes resulted in the highest (($p<0.05$) from the 30 minutes and did not differ significantly from the 60 minute cooling) mean recovery fraction and 240 minutes had the second highest (($p<0.05$) from the 30 minute and tended to differ from 60 minute period) mean recovery fraction.

With 7 h of glycerol equilibration, 30 (0.36±0.15) and 120 (0.47±0.07) minutes, as well as 30 and 240 (0.49±0.06) minutes of cooling showed significant differences ($p<0.05$), when comparing the respective mean recovery fractions. The differences between fractions resulting from 60, 120 and 240 minute periods, as well as the difference in mean fraction from 30 and 60 minutes of cooling were not significantly different. The period of 240 minutes once again resulted in the highest (($p<0.05$), compared to the 30 minute period) mean recovery fraction, with the fraction values decreasing for periods of 120, 60 and 30 minutes of cooling.

When combined with a glycerol equilibration period of 8 h, no significant differences between the mean fractions resulting from the different cooling periods were recorded. Cooling semen for 60 minutes resulted in a slightly lower (not significant) mean recovery fraction (0.42±0.12) than when semen was cooled for 30 minutes (0.43±0.13). The highest (although not significantly different values) (0.49±0.10) and second highest mean fractions (0.46±0.09) were recorded for 240 and 120 minutes, respectively.

4.6.2. Effects of different glycerol equilibration periods on the recovery fractions of bovine semen

When comparing the different glycerol equilibration periods over all cooling periods, 4 h of equilibration yielded the lowest ($p<0.05$) mean recovery fraction (0.34±0.16). Increasing recovery fractions, recorded for 5 h (0.39±0.15), 6 h (0.43±0.13) and 7 h (0.44±0.12) while 8 h of equilibration resulted in the highest (not significantly different from 6 and 7 h, although differed from the 4 and 5 h period; $p<0.05$ with a recovery fraction of 0.45±0.11).The mean recovery fraction after 4 h differed significantly ($p<0.05$) from the recovery fractions recorded after all 4 other equilibration periods. A glycerol equilibration period of 5 h yielded an mean recovery fraction, that differed significantly ($p<0.05$) from the recovery fractions after 7 and 8 h. It tended to differ from the recovery fraction after 6 h. The recovery fractions following glycerol equilibration periods of 6, 7 and 8 h were not significantly different from one another.

With a cooling period of 30 minutes, significant differences ($p<0.05$) were recorded between the mean recovery fractions for glycerol equilibration periods of 4 h (0.27±0.17) and 6 h (0.36±0.15) h, 4 h and 7 h (0.36±0.15) h, 4 h and 8 h (0.43±0.13). Significant differences ($p<0.05$) also existed between the mean recovery fractions resulting after equilibration periods of 5 h (0.27±0.17) and 6 h, as well as 5 h and 8 h periods. The difference between the mean recovery fractions at 5 and 7 h glycerol equilibration periods only tended to differ. Equilibration periods of 4 h (0.27±0.17) and 5 h (0.27±0.17) both yielded the lowest ($p<0.05$) mean recovery fractions. The periods of 6 and 7 h resulted in mean recovery fractions of 0.36±0.15 and 0.36±0.15, while a period of 8 h (0.43±0.13) resulted in the highest ($p<0.05$) recovery fraction. The differences between the mean recovery fractions of 4 and 5 h,

as well as 6 and 7 h, 6 and 8 h and 7 and 8 h of equilibration were not significantly different.

For the 60 minutes cooling period, the recovery fraction after 4 h of glycerol equilibration (0.25±0.16) was significantly lower ($p<0.05$) than the mean recovery fractions recorded after 5 h (0.39±0.14), 6 h (0.40±0.15), 7 h (0.43±0.12) and 8 h (0.42±0.12) of equilibration. The mean recovery fractions after equilibration periods of 5, 6, 7 and 8 h were not different. A period of 5 h recorded the second lowest (not significant) mean recovery fraction, followed by the 6 h period, while 7 and 8 h of equilibration recorded the highest (not significantly different from 5,6 or 8 h) and second highest (not significantly different from 5, 6 or 7 h) recovery fractions.

With a cooling period of 120 minutes, there was a significant difference ($p<0.05$) between the mean recovery fractions of 4 h (0.38±0.13) and 6 h (0.49±0.08) h of equilibration while periods of 4 and 7 h (0.47±0.07), as well as 4 and 8 h (0.46±0.089) only tended to differ. The differences between the mean recovery fractions after 5, 6, 7 and 8 h of equilibration were not significant. The lowest (differed significantly ($p<0.05$) from 6 h and only tended to differ for 7 and 8 h periods) recovery fraction, was once again the result of 4 h of glycerol equilibration. Periods of 5, 8 and 7 h resulted in increasing fractions. After a cooling period of 120 minutes, an equilibration period of 6 h yielded the highest (not different from 5, 7 or 8 h, differed significantly from 4 h, $p<0.05$) recovery fraction.

Following a cooling period of 240 minutes, there were no significant differences between the mean recovery fractions resulting from the respective glycerol equilibration periods. The mean recovery fractions increased with longer equilibration periods, with 4, 5, 6, 7 and 8 h of equilibration resulting in a mean recovery fraction of 0.46±0.10, 0.47±0.10, 0.48±0.07, 0.49±0.06 and 0.49±0.10, respectively.

TABLE 4.6: The overall effects of different cooling and glycerol equilibration periods on the mean (±SD) recovery fraction of bovine semen

GLYCEROL EQUILIBRATION PERIOD (hrs)	COOLING PERIOD (min) 30	60	120	240	Mean±SD
4	0.27^{a} ±0.17	0.25^{a} ±0.16	0.38^{jA} ±0.13	0.46^{jBl} ±0.10	**0.34$^{\underline{a}}$ ±0.16**
5	0.27^{abA} ±0.17	0.39^{gA} ±0.14	0.42^{gjk} ±0.10	0.47^{gBl} ±0.10	**0.39$^{\underline{bA}}$ ±0.15**
6	0.36^{c} ±0.15	0.40^{cghA} ±0.15	0.49^{hBk} ±0.08	0.48^{hBl} ±0.07	**0.43$^{\underline{bB,c}}$ ±0.13**
7	0.36^{bBcd} ±0.15	0.43^{dgi} ±0.12	0.47^{ijBk} ±0.07	0.49^{il} ±0.06	**0.44$^{\underline{c}}$ ±0.12**
8	0.43^{ce} ±0.13	0.42^{eg} ±0.12	0.46^{ejBk} ±0.09	0.49^{el} ±0.10	**0.45$^{\underline{c}}$ ±0.11**
Mean ±SD	**0.34$^{\underline{a}}$ ±0.16**	**0.38$^{\underline{b}}$ ±0.15**	**0.44$^{\underline{cA}}$ ±0.10**	**0.48$^{\underline{cB}}$ ±0.09**	

a,b,c Means in the same row or column with different superscripts differ significantly ($p<0.05$)
a,b,c Means in the same row or column with different superscripts differ significantly ($p<0.05$)
A.B Means in the same row or column with different superscript letter tended to differ ($p<0.1$)
A.B Means in the same row or column with different superscript letter tended to differ ($p<0.1$)

Figure 4.7: The relationship between cooling period and the average recovery fraction of cryopreserved bovine semen

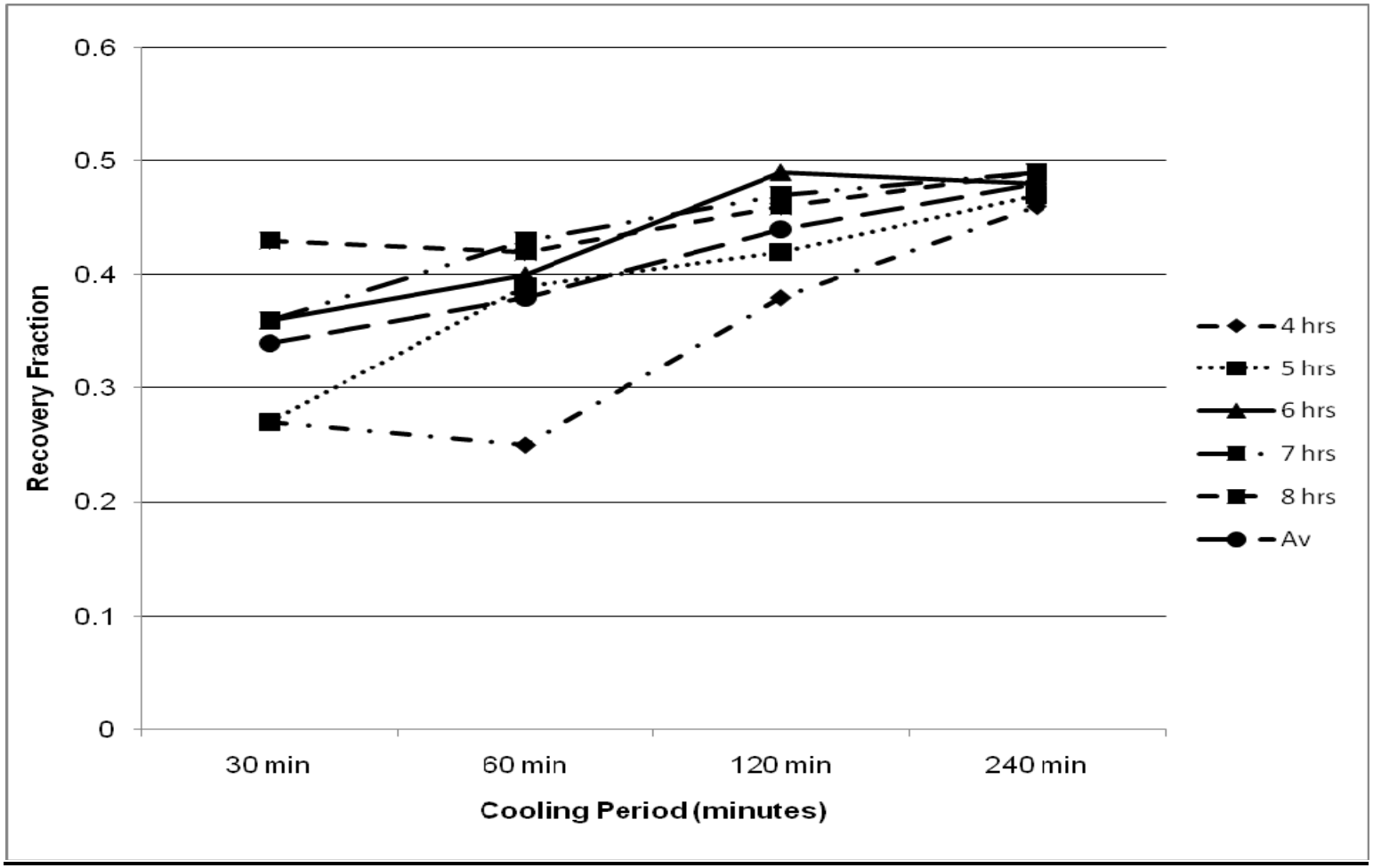
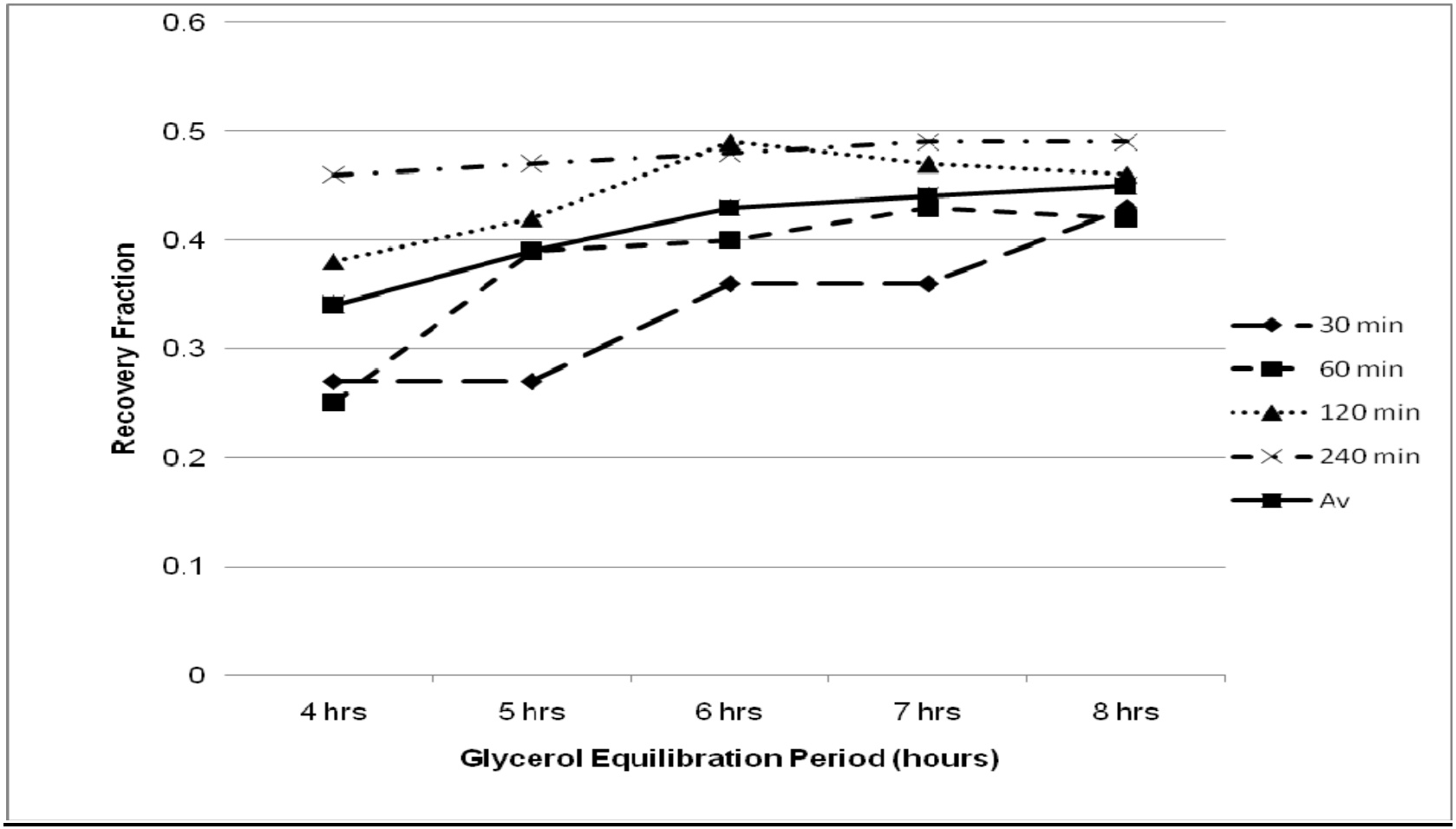


Figure 4.8: The relationship between glycerol equilibration period and the average recovery fraction of cryopreserved bovine semen



4.7. Effect of different cooling periods on the post-thaw motility of Boran and Nguni semen

The effect of the cooling interval on the post-thawing sperm motility is set out in Table 4.7. The general trend regarding the effect of the different cooling periods on post-thaw sperm motility rate of the Boran semen is illustrated in Figure 4.9. Similarly the trend for the effect of different cooling periods for Nguni semen is illustrated in Figure 4.10.

The author could not find any previous studies comparing frozen-thawed semen from Boran and Nguni bulls.

4.7.1. Effect of different cooling periods on the post-thaw sperm motility of Boran semen

When comparing the different cooling periods over all the equilibration periods, generally longer periods of cooling resulted in a higher post-thaw sperm motility. Cooling of semen from Boran bulls for periods of 30, 60, 120 and 240 minutes yielded a mean post-thaw sperm motility of 25.7±13.9%, 30.3±11.4%, 35.2±5.3% and 37.2±5.5%, respectively. No significant difference between the post-thaw motility rates when comparing cooling periods of 30 and 60, as well as 120 and 240 minutes were recorded. Significant differences ($p<0.05$) were recorded between the post-thaw motility after 30 and 120, 30 and 240, and also after 60 and 240 minutes. Cooling periods of 60 and 120 minutes recorded post-thaw motility rates that tended to differ from one another.

Ennen *et al.* (1976) reported a cooling period of either 120 or 240 minutes to be superior to a cooling period of 30 minutes. Although the results from the present study did not significantly differ, there was an increase in the post-thaw motility percentages, as the cooling period was increased from 30 to 240 minutes.

Dhami and Sahni (1993) reported that a 2 h cooling period yielded significantly higher ($p<0.01$) mean post-thaw sperm motility results, than a 1 h cooling period. This is in agreement with the results from the present study, which also found a slight difference between the mean post-thaw motility rates (for the Boran breed) following a 1 or 2 h cooling period.

When comparing the different cooling periods at 4 h of glycerol equilibration, increasing the cooling period resulted in higher mean post-thaw motility rates. A 30 minute cooling period produced a mean post-thaw motility rate of 17.8±13.3%, while when cooling semen from the Boran bulls for 60, 120 and 240 minutes resulted in post-thaw motility of 20.2±15.1%, 31.0±8.9% and 35.8±8.0%, respectively. Significant differences ($p<0.05$) were recorded between the mean post-thaw motility after 30 and 240 minutes, as well as after 60 and 240 minutes. The Boran recorded another significant difference ($p<0.05$) between the mean post-thaw motility following cooling periods of 30 and 120 minutes, as well as a tendency towards a difference, when comparing 60 and 120 minutes of cooling. The resulting mean post-thaw motility rates after 30 and 60 minutes, as well as after 120 and 240 minutes were not significantly different from one another.

After 5 h of glycerol equilibration followed the various cooling periods, the mean post-thaw sperm motility increased when the cooling periods of 30 (20.7±15.6%), 60 (30.0±11.9%), 240 (35.2±6.8%) and 120 minutes (36.2±5.3%) were arranged in that order. The differences in mean post-thaw motility rate after 30 and 120 and also 30 and 240 minutes were significantly different ($p<0.05$) from each other. Post-thaw motility rates yielded following 60, 120 and 240 minutes, as well as 30 and 60 minutes were not significantly different.

When the various cooling periods were followed by a glycerol equilibration period of 6 h, the differences between the respective mean post-thaw motility rates for the Boran, were not different. A cooling period of 30 minutes produced the lowest (not significant) mean post-thaw motility (28.7±12.8%) followed by periods of 60 (33.2±7.0%), 120 (37.3±2.8%) and 240 minutes (37.5±2.7%).

With 7 h of glycerol equilibration, the cooling periods of 30, 60, 120 and 240 minutes produced a mean post-thaw sperm motility of 28.5±14.4%, 33.5±12.2%, 36.0±1.5% and 39.2±3.4%. The difference between the mean post-thaw motility after 30 and 240 minute cooling periods tended to differ. The respective mean post-thaw motility rates after cooling periods of 60, 120 and 240 minutes were not significantly different. The mean post-thaw motility after cooling periods of 30 and 60, as well as 30 and 120 minutes were not significantly different.

When combining the cooling periods of 30 (33.0±11.9%), 60 (34.7±4.5%), 120 (35.7±3.7%) and 240 minutes (38.5±5.8%) with an 8 h glycerol equilibration period, produced progressively higher mean post-thaw motility rates. The differences between the mean post-thaw sperm motility rates were not significantly significant.

4.7.2. Effects of different cooling periods on the post-thaw sperm motility of Nguni semen

The mean post-thaw sperm motility over all equilibration periods were 31.7±14.1%, 34.4±14.5%, 40.0±11.0% and 44.1±8.0% for cooling periods of 30, 60, 120 and 240 minutes. These mean post-thaw sperm motility again increased as the period of cooling increased. Significant differences were recorded ($p<0.05$) between the mean post-thaw motility for cooling periods of 30 and 120, 30 and 240, 60 and 120, as well as 60 and 240 minutes. Cooling periods of 120 and 240 minutes resulted in mean post-thaw motility rates that tended to differ. The mean rates after 30 and 60 minutes of cooling were not significantly different.

Dhami and Sahni (1993) reported that a 2 h cooling period yielded significantly higher ($p<0.01$) mean post-thaw motility, than a 1 h cooling period. This is in agreement with the results of the present study, which also found a significant difference ($p<0.1$) between the mean post-thaw motility rates (for the Nguni breed), recorded by a 1 or 2 h cooling period.

Ennen *et al.* (1976) recorded a cooling period of either 120 or 240 minutes to be superior to a cooling period of 30 minutes. In the present study there was also a significant difference ($p<0.05$) in the mean post-thaw sperm motility resulting from the shorter cooling periods of either 30 or 60 minutes and the longer cooling periods (120 or 240 minutes).

When comparing the different cooling periods at 4 h of glycerol equilibration, the 60 minute period resulted in the lowest ($p<0.05$ from 120 and 240 minutes) mean post-thaw motility rates (22.8±13.6%). When using cooling periods of 30 (27.2±15.8%), 120 (33.2±13.4%) and 240 minutes (42.4±9.3%), the mean post-thaw motility progressively increased. The Nguni bulls recorded significant differences ($p<0.05$) between the mean post-thaw motility rates after 60 and 120 minutes, as well as after 60 and 240 minutes. There was also a significant difference ($p<0.05$) between the

mean post-thaw motility rates resulting from cooling periods of 30 and 240 minutes. The mean post-thaw motility rates from 120 and 240 minutes of cooling only tended to differ. No significant differences between the mean post-thaw motility rates after cooling for 30 and 60 minutes, as well as after 30 and 120 minutes were recorded.

When using 5 h of glycerol equilibration, followed by cooling periods of 240 (44.9±8.0%), 60 (36.7±13.6%), 120(35.3±11.1%) and 30 (25.4±14.5%) minutes, the mean post-thaw motility declined. The Nguni recorded significant differences ($p<0.05$) between the mean post-thaw sperm motility rates after 30 and 60 minutes, as well as 30 and 240 minutes. The Nguni mean post-thaw motility tended to differ after cooling times of 30 and 120 minutes, as well as 120 and 240 minutes. The mean post-thaw motility rates after cooling periods of 60 and 120 minutes, as well as 60 and 240 minutes, were not significantly different.

When a glycerol equilibration period of 6 h was implemented, followed by cooling periods of 30 (33.1±13.8%), 60 (35.7±16.5%), 240 (44.8±7.6%) or 120 (44.9±7.4%) minutes, the mean post-thaw motility rates increased. There were significant differences ($p<0.05$) between the mean post-thaw motility, that resulted from cooling periods of 30 and 120 minutes, as well as 30 and 240 minutes. When comparing the Nguni mean post-thaw motility after 60 minutes to the mean post-thaw motility rates after 120 and 240 minutes respectively, both comparisons tended to differ. The mean post-thaw motility rates after 120 and 240 minutes were not significantly different.

Following 7 h of glycerol equilibration, cooling periods of 30 (32.6±13.1%), 60 (39.6±9.9%), 120 (43.6±8.6%) and 240 (44.4±6.5%) minutes produced mean post-thaw sperm motility rates that increased, as the intervals increased. The Nguni recorded significant differences ($p<0.05$) between the mean post-thaw motility after 30 and 120 minutes, as well as 30 and 240 minutes of cooling. The mean post-thaw motility rates after cooling periods at 30 and 60, as well as periods of 60 and 120 or 60 and 240 minutes, were not significantly different.

Cooling periods of 30, 60, 120 and 240 minutes followed by a 8 h glycerol equilibration period produced mean post-thaw sperm motility rates that were not

significantly different from one another. These post-thaw motility rates recorded were 40.2±11.1%, 37.3±15.0%, 43.1±10.2% and 43.9±9.7% respectively.

4.7.3. Effects of different cooling periods and breed on the post-thaw sperm motility of bovine semen

Over all equilibration periods, cooling periods of 30 and 240 minutes resulted in a mean post-thaw motility that differed significantly ($p<0.05$) between the breeds (25.7±13.9% and 37.2±5.5% for the Boran, with a higher mean post-thaw motility of 31.7±14.1% and 44.1±8.00% for the Nguni). Cooling periods of 60 and 120 minutes yielded increasing mean post-thaw motility rates for both the Boran (30.3±11.4% and 35.2±5.3%) and Nguni bulls (34.4±14.5% and 40.0±11.0%).

When followed by a 4 h glycerol equilibration period, the mean post-thaw motility rates of the Boran and Nguni bulls after cooling periods of 30, 60, 120 and 240 minutes, were not significantly different. The mean post-thaw motility rate after 30 minutes of cooling was 17.8±13.3% for the Boran, and 27.2±15.8% for the Nguni. A 60 minute cooling period yielded mean post-thaw motility rates of 20.2±15.1% (Boran) and 22.8±13.6% (Nguni). Cooling semen for 120 minutes, resulted in mean post-thaw sperm motility rates of 31.0±8.9% and 33.2±13.4% for the Boran and Nguni respectively. A cooling period of 240 minutes yielded a Boran mean post-thaw motility rate of 35.8±8.0% and an Nguni mean of 42.4±9.3%.

When combining a 5 h glycerol equilibration period with cooling periods of 30, 60 or 120 minutes it resulted in the mean post-thaw motility not being significantly different between the two breeds. The mean Boran post-thaw motility rate after 30 minutes of cooling was 20.7±15.6% and the Nguni 25.4±14.5%, respectively. Applying a 60 minute cooling period yielded an mean post-thaw motility of 30.0±11.9% for the Boran and 36.7±13.6% for the Nguni. Mean post-thaw motility rates resulting from a 120 minute cooling period were 36.2±5.4% and 35.3±11.1% for the Boran and Nguni. The 240 minute cooling period yielded a mean post-thaw motility that tended to differ ($p<0.1$). These mean post-thaw motility rates were 35.2±6.8% for the Boran and 44.9±8.0% for the Nguni.

After glycerol equilibration for a period of 6 h, no significant differences were observed regarding the mean post-thaw motility of the two breeds, with cooling

periods of either 30 (28.7±12.8% Boran and 33.1±13.8% Nguni) or 60 (33.2±7.0% Boran and 35.7±16.5% Nguni) minutes. When cooling periods of 120 or 240 minutes were used, again no significant differences between the breed mean post-thaw motility rates were recorded. The mean post-thaw sperm motility rates resulting from a 120 minute cooling period were 37.3±2.8% for the Boran and 44.9±7.4% for the Nguni. The mean post-thaw motility yielded for a 240 cooling period was 37.5±2.7% for the Boran, and 44.8±7.6% for the Nguni bulls.

Equilibrating extended semen for 7 h after the various cooling periods resulted in no significant differences between the breed mean post-thaw motility rates (36.0±1.5% and 39.2±3.4% for the Boran and 43.6±8.6% and 44.4±6.5% for the Nguni), when combined with cooling periods of 120 and 240 minutes. Cooling periods of 30 (Boran 28.5±14.4% and Nguni 32.6±13.1%) and 60 (Boran 33.5±12.2% and Nguni 39.6±9.9%) minutes also resulted in mean post-thaw motility rates not being significantly different between the two breeds.

With an 8 h equilibration period, the respective cooling periods yielded mean post-thaw motility rates that were not significantly different between the breeds. A cooling period of 30 minutes resulted in mean post-thaw motility rates of 33.0±11.9% (Boran) and 40.2±11.1% (Nguni), while a period of 60 minutes yielded a mean post-thaw sperm motility of 34.7±4.5% (Boran) and 37.3±15.0% (Nguni). The mean post-thaw motility rates of 35.7±3.7% (Boran) and 43.1±10.2% (Nguni) were recorded a 120 minute cooling period, while a slightly higher mean post-thaw motility of 38.5±5.8% (Boran) and 43.9±9.7% (Nguni) resulted following cooling for 240 minutes.

TABLE 4.7: The between and within breed effects of different combinations of cooling and glycerol equilibration periods on the mean (±SD) post-thaw sperm motility of bovine semen

GLYCEROL EQUILIBRATION TIME (hrs)	BREED	COOLING PERIOD (min) 30	60	120	240
4	B	17.8^{a}±13.3	20.2^{abA}±15.1	31.0^{bBc}±8.9	35.8^{c}±8.0
	N	27.2^{ab}±15.8	22.8^{a}±13.6	33.2^{bcA}±13.4	42.4^{cB}±9.3
5	B	20.7^{a}±15.6	30.0^{ab}±11.9	36.2^{b}±5.3	$_{aA}35.2^{b}$±6.8
	N	25.4^{aA}±14.5	36.7^{b}±13.6	35.3^{aBbA}±11.1	$_{aB}44.9^{bB}$±8.0
6	B	28.7^{a} ±12.8	33.2^{a}±7.0	37.3^{a}±2.8	37.5^{a}±2.7
	N	33.1^{a}±13.8	35.7^{abA}±16.5	44.9^{bB}±7.4	44.8^{bB}±7.6
7	B	28.5^{aA}±14.4	33.5^{a}±12.2	36.0^{a}±1.5	39.2^{aB}±3.4
	N	32.6^{a}±13.1	39.6^{ab}±9.9	43.6^{b}±8.6	44.4^{b}±6.5
8	B	33.0^{a}±11.9	34.7^{a}±4.5	35.7^{a}±3.7	38.5^{a}±5.8
	N	40.2^{a}±11.1	37.3^{a}±15.0	43.1^{a}±10.2	43.9^{a}±9.7
Overall mean (±SD)	B	**$_{a}25.7^{a}$ ±13.9**	**$_{a}30.3^{abA}$ ±11.4**	**$_{aA}35.2^{bBc}$ ±5.3**	**$_{a}37.2^{c}$ ±5.5**
	N	**$_{b}31.7^{a}$ ±14.1**	**$_{a}34.4^{a}$ ±14.5**	**$_{aB}40.0^{bA}$ ±11.0**	**$_{b}44.1^{bB}$ ±8.00**

^{a,b,c} Means in the same row with different superscripts differ significantly ($p<0.05$)
$_{a, b}$ Means in the same cooling and equilibration period with different subscripts differ significantly ($p<0.05$)
$^{aA.aB}$Means in the same row with different superscripts tended to differ ($p<0.1$)
$_{aA,aB}$ Means in the same cooling and equilibration period with different subscripts tended to differ ($p<0.1$)
^{a,b,c} Means in the same column with different superscripts differ ($p<0.05$)
$^{aA.aB}$ Means in the same column with different superscripts tended to differ ($p<0.1$)
B Boran
N Nguni

Figure 4.9: The relationship between the cooling period and average post-thaw sperm motility of cryopreserved semen of Boran bulls

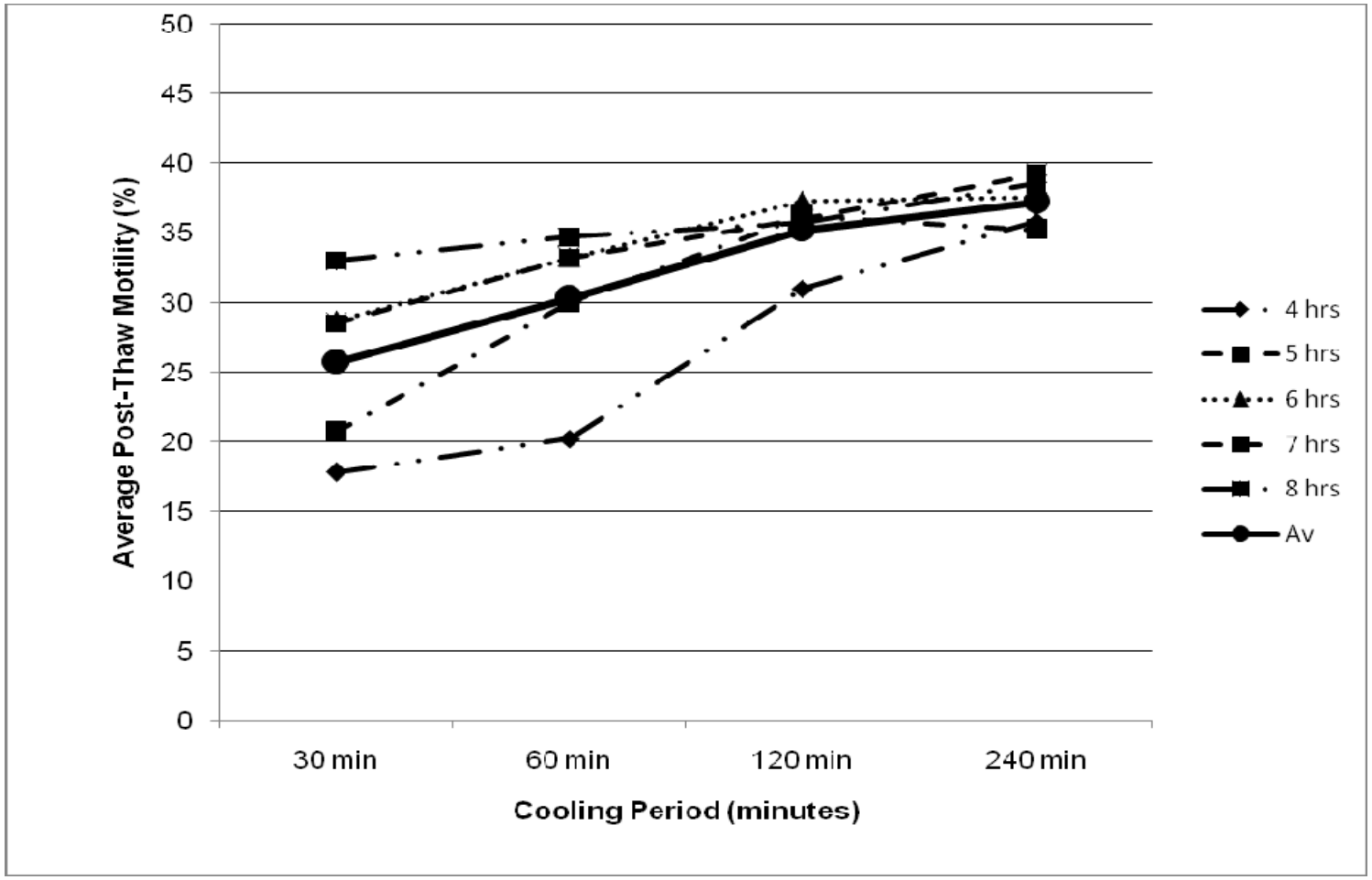
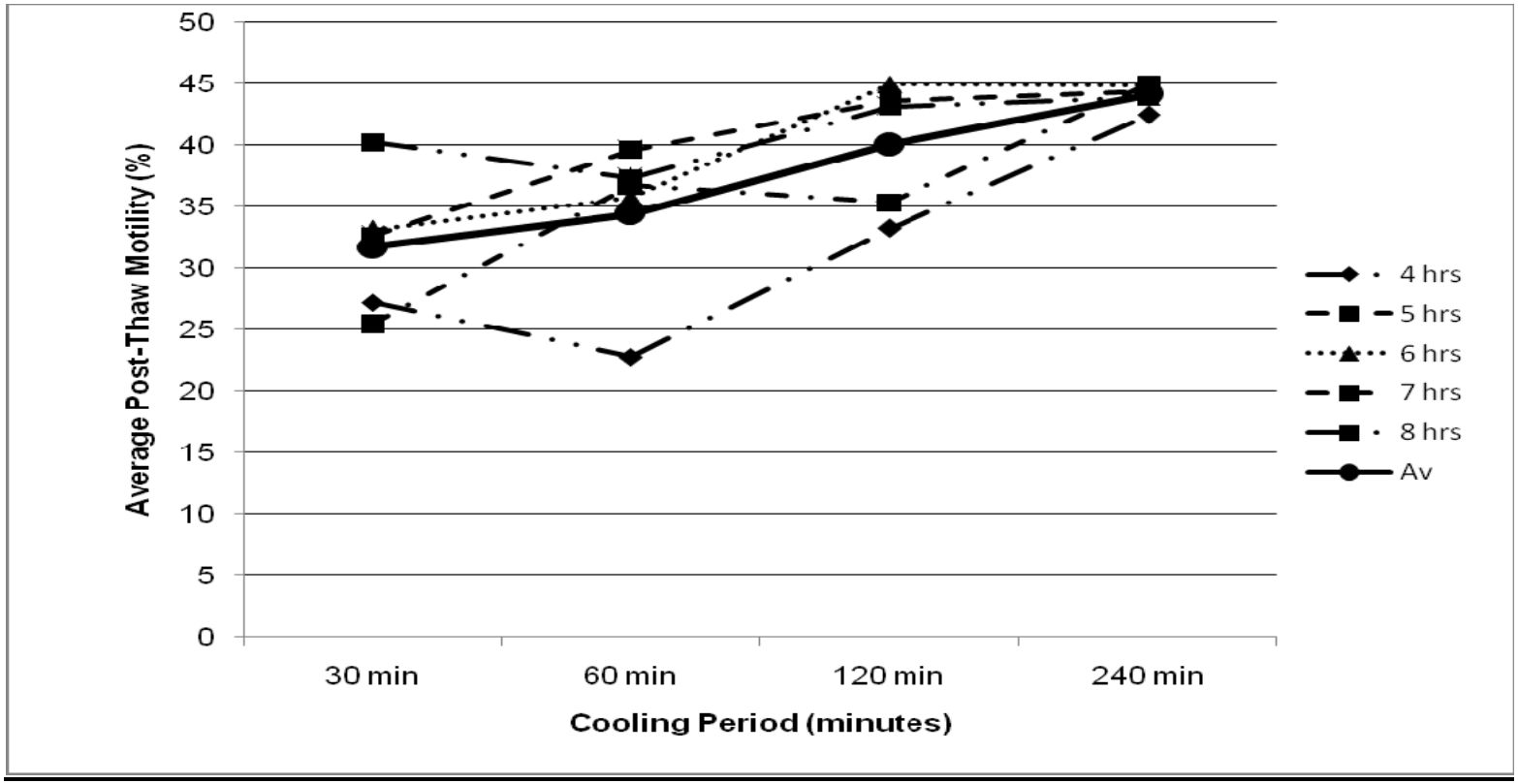


Figure 4.10: The relationship between the cooling period and average post-thaw sperm motility of cryopreserved semen of Nguni bulls



4.8. Effect of different cooling periods on the recovery fraction of Boran and Nguni semen

The effect of different cooling periods on the recovery fraction is summarized in Table 4.8. The general trend of the effect of different cooling periods on the mean recovery fraction for Boran semen is illustrated in Figure 4.11, while that for the Nguni semen is illustrated in Figure 4.12.

The author could not find any previous studies using mean recovery fraction of bovine semen as a variable. Recovery fraction (% post-thaw motility / % initial motility) is a more accurate measurement of the effect of the cryopreservation process on sperm motility. The author could also not find any previous studies comparing frozen-thawed semen from Boran and Nguni bulls.

4.8.1. Effects of different cooling periods on the recovery fraction of Boran semen

Over all glycerol equilibration periods, the cooling of semen for 30, 60, 120 and 240 minutes yielded increasing recovery fractions of 0.32±0.17, 0.37±0.14, 0.43±0.07 and 0.45±0.07. Significant differences ($p<0.05$) being recorded between the mean recovery fractions, when comparing the cooling periods of 30 and 120, 30 and 240 and also 60 and 240 minutes. The mean recovery fractions for cooling periods of 30 and 60 minutes or periods of 60 and 120 minutes tended to differ but not significantly. Means from cooling periods of 120 and 240 minutes were not significantly different from one another.

When comparing the different cooling periods following 4 h of glycerol equilibration, the Boran recorded significant differences ($p<0.05$) between the mean recovery fractions after 30 and 240 minutes, as well as 60 and 240 minutes. There was another significant difference ($p<0.05$) between the mean recovery fractions from cooling periods of 30 and 120 minutes as well as a tendency toward significant difference ($p<0.1$) when comparing 60 and 120 minutes of cooling. The mean recovery fraction increased as cooling period lengthened. Thus, a period of 30 minutes produced the lowest ($p<0.05$, for 120 and 240 minutes) mean recovery fraction (0.22±0.17), followed by periods of 60 (0.25±0.19), 120 (0.38±0.11) and 240 minutes (0.43±0.10).

Cooling periods of 30 and 120 minutes resulted in the lowest ($p<0.05$; for 120 and 240 minutes) (0.25±0.19) and the highest ($p<0.05$; for 30 minutes) (0.44±0.08) mean recovery fractions, when combined with 5 h of glycerol equilibration. Periods of 60 and 240 minutes recorded the second highest (not significant) (0.37±0.15) and second lowest (not significant) (0.42±0.09) recovery fractions. The differences in mean recovery fraction recorded after 30 and 120 and also 30 and 240 minutes, were significant ($p<0.05$). The differences between mean recovery fractions after cooling periods of 60, 120 and 240 minutes as well as after 30 and 60 minutes were not significantly different.

When the various cooling periods were followed with a glycerol equilibration period of 6 h, both the 120 and 240 minutes of cooling produced the highest (not significantly different) mean recovery fraction (0.45±0.05). A period of 30 minutes resulted in the lowest (not significantly different) mean recovery fraction (0.35±0.16), while a 60 minute period yielded the second lowest (not significantly different (0.40±0.09). The differences between the respective mean recovery fractions for the Boran were not significantly different.

With 7 h of glycerol equilibration, a longer cooling period produced a higher mean recovery fraction. Periods of 30, 60, 120 and 240 minutes resulted in mean recovery fractions of 0.35±0.18, 0.41±0.15, 0.43±0.03 and 0.47±0.05, respectively. The Boran tended to differ, when comparing the mean recovery fractions after 30 and 240 minutes of cooling. The differences between the respective mean recovery fractions of the Boran after cooling periods of 30, 60 and 120 minutes as well as after 60 and 240 minutes and also 120 and 240 minutes were also not significantly different.

When an 8 h glycerol equilibration was preceded by cooling periods of 30, 60, 120 and 240 minutes, the mean recovery fractions increased. The lowest (not significantly different) recovery fraction (0.40±0.15) was the result of a 30 minute cooling period while the 240 minutes of cooling resulted in the highest (not significantly different) recovery fraction (0.46±0.08). Periods of 60 and 120 minutes yielded a mean recovery fraction of 0.42±0.06 and 0.43±0.05, respectively. No significant differences between the respective mean recovery fractions of the Boran were recorded.

4.8.2. Effects of different cooling periods on the recovery fraction of Nguni semen

Mean recovery fractions of 0.36±0.15, 0.39±0.16, 0.46±0.12 and 0.51±0.08 were the results of cooling semen for 30, 60, 120 and 240 minutes respectively, over all glycerol equilibration periods. The mean recovery fractions after 30 and 60 minutes of cooling were not significantly different from one another. There were however significant differences ($p<0.05$) between the recovery fractions after cooling periods of 30 and 120, 30 and 240, 60 and 120, as well as 60 and 240 minutes. Cooling semen for periods of either 120 or 240 minutes resulted in mean recovery fractions that tended to differ from one another.

When comparing the different cooling periods at 4 h of glycerol equilibration, periods of 240 and 60 minutes cooling yielded the highest (0.49±0.09) and lowest (0.26±0.15) mean recovery fractions. The Nguni recorded significant differences ($p<0.05$) between the mean recovery fractions resulting from cooling periods of 30 (0.31±0.18) and 240; 60 and 240 minutes, as well as 60 and 120 (0.38±0.14) minutes. The mean recovery fractions from 120 and 240 minutes of cooling was slightly different. There was no significant difference between the mean recovery fractions resulting for cooling periods of 30 and 120 minutes.

When 5 h of glycerol equilibration followed the various cooling periods, the Nguni recorded a significantly ($p<0.05$) smaller fraction after 30 minutes (0.29±0.16), compared to cooling periods of 120 (0.41±0.12), 60 (0.42±0.14) and 240 (0.52±0.90) minutes, that produced progressively higher recovery fractions. The Nguni also tended to differ in the mean recovery fractions, when comparing 240 minutes to both 60 and 120 minutes. There was no significant difference between the mean recovery fractions resulting from cooling periods of 60 and 120 minutes.

When cooling periods of 30, 60, 120 and 240 minutes were followed by a glycerol equilibration period of 6 h, the resulting mean recovery fractions were 0.38±0.15; 0.41±0.18; 0.52±0.08 and 0.52±0.07. The mean recovery fractions improved with longer cooling periods. There were also significant differences ($p<0.05$) between mean recovery fractions from cooling periods of 30 and 120 minutes, as well as 30 and 240 minutes. When comparing the Nguni mean recovery fraction for 60 minutes

to the mean recovery fractions of 120 and 240 minutes respectively, both comparisons tended to differ in the respective mean recovery fractions. No significant difference between the mean recovery fractions resulting for the cooling periods of 30 and 60 minutes as well as 120 and 240 minutes were recorded.

With 7 h of glycerol equilibration, the Nguni recorded significant differences ($p<0.05$) between the mean fractions after 30 and 120 minutes, as well as 30 and 240 minutes of cooling. The mean recovery fraction steadily increased as the length of the cooling period increased. The lowest ($p<0.05$ from 120 and 240 minutes cooling) mean recovery fraction (0.38±0.14) was the result of a 30 minute cooling period. The highest ($p<0.05$ from 30 minutes cooling) fraction (0.52±0.07) was obtained when using a 240 minute cooling period. Periods of 60 and 120 minutes of cooling produced mean fractions of 0.46±0.09 and 0.50±0.08 each. Mean recovery fractions resulting from cooling periods of 60, 120 and 240 minutes, as well as 30 and 60 minutes were not significantly different from each other

The different cooling periods yielded no significant differences between the respective mean recovery fractions of the Nguni following 8 h of glycerol equilibration. The combination of a 60 minute cooling period and 8 h of equilibration resulted in the lowest (not significantly different) mean recovery fraction (0.43±0.16). When the cooling period was lengthened to 240 minutes the resulting mean fraction was the highest (not significantly different) (0.51±0.11). Cooling periods of 30 and 120 minutes yielded mean recovery fractions of 0.46±0.11 and 0.50±0.10.

4.8.3. Effects of different cooling periods and breed on the recovery fraction of bovine semen

When cooling periods between the breeds were compared over all equilibration periods, there were no significant differences between the mean recovery fractions after 60 or 120 minutes of cooling. The mean recovery fractions recorded were 0.37±0.14 and 0.43±0.07 for the Boran, while the Nguni recorded recovery fractions of 0.39±0.16 and 0.46±0.12. Cooling semen for 240 minutes before equilibration resulted in a significant difference ($p<0.05$) between the mean recovery fractions for the Boran (0.45±0.07) and Nguni (0.51±0.08) breeds. The mean recovery fractions of the Boran and Nguni bulls resulting after a cooling periods of 30 minutes were

slightly different. The Boran recorded a mean recovery fraction of 0.32±0.17, while the Nguni recorded a mean recovery fraction of 0.36±0.15.

There were no significant differences recorded between the mean recovery fractions for the different breeds when an equilibration period of 4 h was allowed. A cooling period of 30 minutes yielded a mean recovery fraction of 0.22±0.17 for the Boran and 0.31±0.18 for the Nguni, while when cooling extended semen for 60 minutes, resulted in mean fractions of 0.25±0.19 and 0.26±0.15. The mean recovery fractions of 0.38±0.11 and 0.43±0.10 (Boran) as well as 0.38±0.14 and 0.49±0.09 (Nguni) were the results of allowing semen to cool for 120 or 240 minutes, respectively.

An equilibration period of 5 h combined with the various cooling periods yielded mean recovery fractions that were not significantly different between the two breeds. Cooling semen for 30, 60, 120 and 240 minutes resulted in mean recovery fractions of 0.25±0.19, 0.37±0.15, 0.44±0.08 and 0.42±0.09 for the Boran, while the same periods of cooling recorded mean fractions of 0.29±0.16, 0.42±0.14, 0.41±0.12 and 0.52±0.09 for the Nguni.

Cooling semen for 30, 60, 120 or 240 minutes, before equilibration for 6 h, yielded mean recovery fractions that were not significantly different between the two breeds. Mean recovery fractions resulting from 30 minutes of cooling were 0.35±0.16 (Boran) and 0.38±0.15 (Nguni), while mean recovery fractions after a 60 minute cooling period were 0.40±0.09 (Boran) and 0.41±0.18 (Nguni). The mean recovery fractions recorded after 120 and 240 minutes were 0.45±0.05 and 0.45±0.05 for the Boran, and 0.52±0.08 and 0.52±0.07 for the Nguni.

With a 7 h equilibration period following cooling periods of 30, 60, 120 or 240 minutes, no significant differences were recorded between the recovery fractions of the two breeds. Cooling periods of 30 or 60 minutes resulted in mean recovery fractions of 0.35±0.18 and 0.41±0.15 for the Boran, as well as 0.38±0.140 and 0.46±0.09 for the Nguni. The mean recovery fractions recorded after 120 minutes of cooling were 0.43±0.03 and 0.50±0.08 for the Boran and Nguni breeds respectively. A cooling period of 240 minutes resulted in a mean recovery fraction of 0.47±0.052 (Boran) and 0.52±0.066 (Nguni).

When combining 8 h of glycerol equilibration with the various cooling periods, no significant differences between the recovery fractions of the breeds were recorded. Cooling semen for periods of 30, 60, 120 or 240 minutes resulted in mean fractions of 0.40±0.15, 0.42±0.06, 0.43±0.05 and 0.46±0.08 (Boran) and 0.46±0.11, 0.43±0.16, 0.50±0.10 and 0.51±0.11 for the Nguni.

TABLE 4.8: The between and within breed effect of different combinations of cooling and glycerol equilibration periods on the mean (±SD) recovery fraction of bovine semen

GLYCEROL EQUILIBRATION TIME (hrs)	BREED	COOLING PERIOD (min) 30	60	120	240
4	B	$0.22^{a}\pm0.17$	$0.25^{abA}\pm0.19$	$0.38^{bBc}\pm0.11$	$0.43^{c}\pm0.10$
	N	$0.31^{ab}\pm0.18$	$0.26^{a}\pm0.15$	$0.38^{bcA}\pm0.14$	$0.49^{cB}\pm0.10$
5	B	$0.25^{a}\pm0.19$	$0.37^{ab}\pm0.15$	$0.44^{b}\pm0.08$	$0.42^{b}\pm0.09$
	N	$0.29^{a}\pm0.16$	$0.42^{bA}\pm0.14$	$0.41^{bA}\pm0.12$	$0.52^{bB}\pm0.09$
6	B	$0.35^{a}\pm0.16$	$0.40^{a}\pm0.09$	$0.45^{a}\pm0.05$	$0.45^{a}\pm0.05$
	N	$0.38^{a}\pm0.15$	$0.41^{abA}\pm0.18$	$0.52^{bB}\pm0.08$	$0.52^{bB}\pm0.07$
7	B	$0.35^{aA}\pm0.18$	$0.41^{a}\pm0.15$	$0.43^{a}\pm0.03$	$0.47^{aB}\pm0.05$
	N	$0.38^{a}\pm0.14$	$0.46^{ab}\pm0.09$	$0.50^{b}\pm0.08$	$0.52^{b}\pm0.07$
8	B	$0.40^{a}\pm0.15$	$0.42^{a}\pm0.06$	$0.43^{a}\pm0.05$	$0.46^{a}\pm0.08$
	N	$0.46^{a}\pm0.11$	$0.43^{a}\pm0.16$	$0.50^{a}\pm0.10$	$0.51^{a}\pm0.11$
Overall Mean (±SD)	**B**	**$_{aA}0.32^{aA}\pm0.17$**	**$_{a}0.37^{aBbA}\pm0.14$**	**$_{a}0.43^{bBc}\pm0.07$**	**$_{a}0.45^{c}\pm0.07$**
	N	**$_{aB}0.36^{a}\pm0.15$**	**$_{a}0.39^{a}\pm0.16$**	**$_{a}0.46^{bA}\pm0.12$**	**$_{b}0.51^{bB}\pm0.08$**

a,b,c Means in the same row with different superscripts differ significantly (p<0.05)

a, b Means in the same cooling and equilibration period with different subscripts differ significantly (p<0.05)

aA.aB Means in the same row with different superscripts tended to differ (p<0.1)

aA,aB Means in the same cooling and equilibration period with different subscripts tended to differ (p<0.1)

a,b,c Means in the same column with different superscripts differ significantly (p<0.05)

aA.aB Means in the same column with different superscripts tended to differ (p<0.1)

B Boran N Nguni

Figure 4.11: The relationship between cooling period and mean recovery fraction of cryopreserved semen for Boran bulls

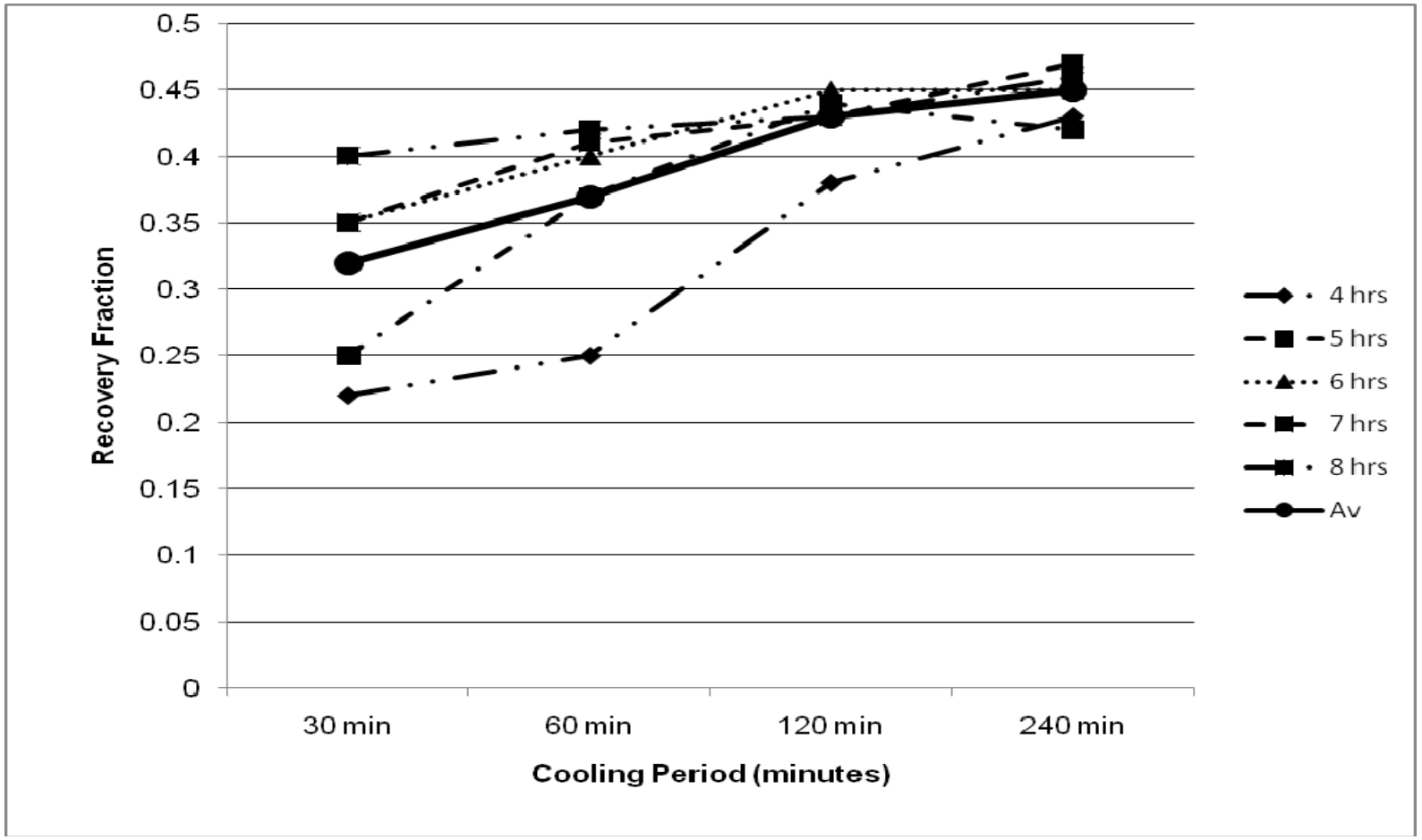
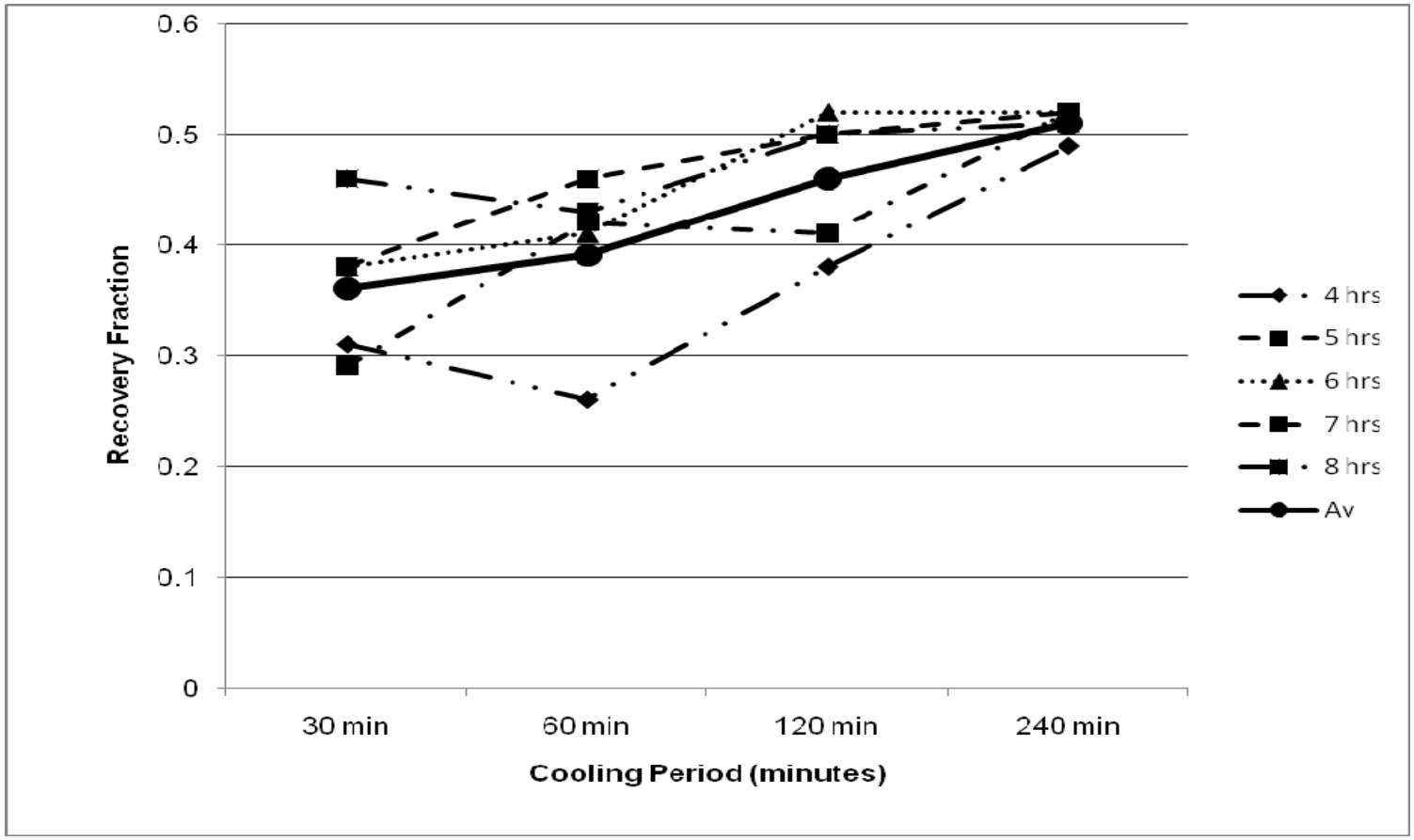


Figure 4.12: The relationship between cooling period and mean recovery fraction of cryopreserved semen for Nguni bulls



4.9. Effect of different glycerol equilibration periods on the post-thaw sperm motility for Boran and Nguni semen

The effect of different glycerol equilibration periods on sperm motility are set out in Table 4.9. The general trend regarding the effect of different glycerol equilibration periods on mean post-thaw motility for Boran semen is illustrated in Figure 4.13, and that of the Nguni semen in Figure 4.14.

The author could not find any previous studies comparing frozen-thawed semen from Boran and Nguni bulls.

4.9.1. Effects of different glycerol equilibration periods on the post-thaw sperm motility of Boran semen

When glycerol equilibration periods were compared over all cooling periods, significant differences ($p<0.05$)were recorded between the mean post-thaw motility rates resulting from 4 and 6, 4 and 7, as well as 4 and 8 h of equilibration. The differences between mean post-thaw sperm motility resulting from 5, 6, 7 and 8 h of glycerol equilibration were not significantly different. The difference between the mean post-thaw motility rates after 4 and 5 h equilibration was also not significantly different. Mean post-thaw motility rates increased with longer periods of equilibration. The mean post-thaw motility rates were 26.2±13.3%, 30.5±11.8%, 34.2±8.0%, 34.3±9.8% and 35.5±7.1% resulting for equilibrating periods of 4, 5, 6, 7 and 8 h respectively.

Wiggin and Almquist (1974) advised against incorporating glycerol equilibration periods exceeding 4 h. This recommendation is in contrast with the results of the present study, which demonstrated that the Boran post-thaw motility after 4 h of glycerol equilibration to be inferior ($p<0.05$) to post-thaw motility resulting from 6, 7 and 8 h.

Tuli *et al.*(1981) reported that a 4 h glycerol equilibration period yielded superior post-thaw motility rates (average 44%), compared to glycerol equilibration periods of either 2 or 6 h. This is not in agreement with the findings of the present study, which found a 6 h glycerol equilibration period to be superior ($p<0.05$) to 4 h of glycerol

equilibration in Boran bulls. Griffin (2004) found glycerol equilibration periods of either 4 or 6 h to be optimum for the freezing of bovine semen.

Herold e*t al.* (2006) studied the effects of glycerol equilibration periods of 2, 3, 4, 5, 6, 7, 8 and 9 h on the post-thaw sperm motility in African buffalo, and found no significant differences between the implementation of different glycerol equilibration periods. In the present study there were significant differences ($p<0.05$) between the post-thaw motility rates after 4 h of glycerol equilibration, compared to glycerol equilibration periods of 6, 7 or 8 h.

With a cooling period of 30 minutes was implemented, the Boran's lowest ($p<0.05$; from 8 h and slightly different from 6 and 7 h) mean post-thaw motility rate (17.8±13.4%) was the result of a 4 h glycerol equilibration period. Increasing of the mean post-thaw motility rates resulted from equilibration periods of 5 (20.7±15.6%), 7 (28.5±14.4%), 6 (28.7±12.8%) and 8 h (33.0±11.9%), respectively. The difference between the mean post-thaw motility after glycerol equilibration periods of 4 and 8 h was significant ($p<0.05$). The mean post-thaw motility rates for the Boran bulls were slightly different after 4 and 6, as well as 4 and 7 h. There was a tendency towards difference between the mean post-thaw motility rates after 5 and 8 h. The mean post-thaw motility rates after 6, 7 and 8 h were not significantly different.

With a cooling period of 60 minutes and 4 h of glycerol equilibration, the Boran recorded a significantly lower ($p<0.05$) mean post-thaw motility (20.2±15.1%) compared to equilibration periods of 6 (33.2±7.0%), 7 (33.5±12.2%) and 8 (34.7±4.5%) h. Although a 5 h equilibration period resulted in a lower mean post-thaw motility rate (30.0±11.9%) when compared to 6, 7 and 8 h, this difference was not significantly different.

Following a 120 minute cooling period, glycerol equilibration periods of 4 (31.0±8.9%), 8 (35.7±3.7%), 7 (36.0±1.6%), 5 (36.2±5.4%) and 6 h (37.3±2.8%) yielded increasing post-thaw motility rates.

After a cooling period of 240 minutes, 5 (35.2±6.8%), 4 (35.8±8.0%), 6 (37.5±2.7%), 8 (38.5±5.8%) and 7 h (39.2±3.4%) of glycerol equilibration yielded increasing post-thaw motility rates.

With cooling period of 120 or 240 minutes, the Boran recorded no significant difference between the mean post-thaw motility for the different equilibration periods.

A study by Ennen *et al.* (1976) to compare the post-thaw sperm motility after cooling for 240 minutes and glycerol equilibration periods of 1, 2, 4, 8 or 16 h recorded no significant difference after glycerol equilibration periods of 1, 2, 4 or 8 h. Graham *et al.* (1956) also found no significant difference between the non-return rates of cows inseminated with semen that was equilibrated with glycerol for either 4 or 8 h. This is in agreement with current results recorded for the Boran in this study, where there were no significant differences between mean post-thaw motility rates after glycerol equilibration periods of 4, 5, 6, 7 or 8 h.

4.9.2. Effect of different glycerol equilibration periods on the post-thaw sperm motility of Nguni semen

The resulting mean post-thaw sperm motility after equilibration periods of 4, 5, 6, 7 and 8 h over all the equilibration periods were 31.4±14.7%, 35.6±13.5%, 39.6±12.7%, 40.0±10.5% and 41.1±11.5% respectively. Significant differences ($p<0.05$) were recorded between the mean post-thaw motility of periods of 4 and 6, 4 and 7 as well as 4 and 8 h. The difference between the post-thaw motility rates after equilibrating for either 5 or 7 h tended to differ. The mean post-thaw sperm motility rates after 6, 7 and 8 h of equilibration were not significantly different from one another. Equilibration of semen for a period of 5 h yielded an mean post-thaw motility that was not significantly different from the post-thaw motility resulting after 4, 6 or 8 h of equilibration.

The effects of glycerol equilibration periods of 2, 3, 4, 5, 6, 7, 8 and 9 h on the post-thaw motility of African buffalo semen and showed significant differences between the different glycerol equilibration periods (Herold e*t al.*, 2006). Griffin (2004) found glycerol equilibration periods of either 4 or 6 h to be optimal for the freezing of bovine semen, while Tuli *et al.*(1981) determined a 4 h glycerol equilibration period to yield superior post-thaw motility percentages, compared to glycerol equilibration periods of either 2 or 6 h. A 2 h glycerol equilibration period was optimum for semen frozen in straws, and that glycerol equilibration periods exceeding 4 h was not recommended by Wiggin and Almquist (1974). In this study semen collected from Jersey, Holstein

and Charolais bulls were used. This is however not in agreement with the results of the present study, which found a 6 h glycerol equilibration period to be superior to 4 h of glycerol equilibration for Nguni bulls.

With a cooling period of 30 minutes, significant differences ($p<0.05$) were recorded between the highest (40.2±11.1%), lowest (25.4±14.5%) and second lowest post-thaw motility (27.2±15.8%), resulting from equilibration periods of 8, 5 and 4 h respectively. The second and third highest mean post-thaw motility rates (resulting from 6 and 7 h of glycerol equilibration) were not significantly different, from either the higher or lower post-thaw motility rates.

With a cooling period of 60 minutes, the lowest mean post-thaw sperm motility (22.8±13.6%), after 4 h, differed significantly ($p<0.05$) from the increasingly higher mean post-thaw motility rates after 6 (35.7±16.5%), 5 (36.7±13.6%), 8 (37.3±15.0%) and 7 (39.6±9.9%) h of equilibration. The mean post-thaw motility rates resulting from glycerol equilibration periods of 5, 6, 7 and 8 h were not significantly different from one another.

For the Nguni, there were significant differences ($p<0.05$) between the mean post-thaw motility rates after 4 (33.2±13.4%) and 6 (44.9±7.4%), as well as 4 and 7 (43.6±8.6%) h of glycerol equilibration, when combined with a cooling period of 120 minutes. The mean Nguni post-thaw motility after 4 and 8 h (43.1±10.2%), as well as 5 (35.3±11.1%) and 6 h only tended to differ. The lower mean post-thaw motility rates (33.2±13.4% and 35.3±11.1%) were associated with the shortest equilibration periods (4 and 5 h), while the longest equilibration period (8 h) resulted in the third highest (tended to differ from 4 h) mean post-thaw motility (43.1±10.2%). Six and 7 h of equilibration were associated with the highest ($p<0.05$; for 4 h and tended to differ from 5 h) (44.9±7.4%) and second highest ($p<0.05$; for 4 h) (43.6±8.6%) mean post-thaw motility rates.

There were no significant differences for the Nguni when a cooling period of 240 minutes was used. Equilibration for periods of 4 (42.4±9.3%), 8 (43.9±9.7%), 7 (44.4±6.5%), 6 (44.8±7.6%) and 5 h (44.9±8.0%) resulted in an increasing mean post-thaw motility rate.

Ennen *et al.* (1976) to comparing the post-thaw motility after cooling for 240 minutes and glycerol equilibration periods of 1, 2, 4, 8 or 16 h found no significant difference after glycerol equilibration periods of 1, 2, 4 or 8 h. This is in agreement with the results found for Nguni semen in this study, where there were no significant differences between the mean post-thaw motility after a cooling period of 240 minutes and glycerol equilibration periods of 4, 5, 6, 7 or 8 h.

4.9.3. Effect of different glycerol equilibration periods and breed on the post-thaw motility rate of bovine semen

When the two breeds were compared over all cooling periods, the mean post-thaw motility rates from the Boran and Nguni after equilibration periods of 4, 5, 6 and 8 h tended to differ, with the Nguni recording higher values. The difference between the breeds after equilibration for 7 h was significant ($p<0.05$) with the mean post-thaw motility for the Boran being 26.2±13.3%, 30.5±11.8%, 34.2±8.0%, 34.3±9.8% and 35.5±7.1% after equilibration for 4, 5, 6, 7 and 8 h. The Nguni had mean post-thaw motility rates of 31.4±14.7%, 35.6±13.5%, 39.6±12.7%, 40.0±10.5% and 41.1±11.5% after the corresponding equilibration periods.

When a cooling period of 30 minutes preceded the various glycerol equilibration periods, there were no significant differences recorded between the post-thaw motility rates recorded for the Boran and Nguni bulls, after each equilibration period. Although there was a general increase in the mean post-thaw sperm motility in both breeds with longer equilibration periods, the Nguni bulls produced post-thaw motility that were consistently higher (not significantly different), than the corresponding Boran bulls, with the same equilibration period. After an equilibration period of 4 h the mean post-thaw motility rates yielded by the Nguni and Boran breeds were 27.2±15.8% and 17.8±13.4% respectively. After 5 h of equilibration the Nguni recorded a mean post-thaw motility of 25.4±14.5% while the Boran was 20.7±15.6%. Similarly the Nguni produced a post-thaw motility of 33.1±13.8%, 32.6±13.1% and 40.2±11.1% after 6, 7 and 8 h of equilibration, while the Boran yielded mean post-thaw motility rates of 28.7±12.8%, 28.5±14.4% and 33.0±11.9% after the same equilibration periods.

There were no significant differences between the mean post-thaw sperm motility rates when a 60 minute cooling period was applied, in combination with the different equilibration periods. The mean post-thaw motility rates recorded for the Nguni breed were once again higher (not significant) than the Boran after corresponding equilibration periods. For the Nguni, periods of 4 h (22.8±13.6%), 6 h (35.7±16.5%), 5 h (36.7±13.6%), 8 h (37.3±15.0%) and 7 h (39.6±9.9%) resulted in increasing post-thaw motility rates. For the Boran equilibration periods of 4 h (20.2±15.1%), 5 h (30.0±11.9%), 6 h (33.2±7.0%), 7 h (33.2±7.0%) and 8 h (34.7±4.6%) resulted in an increasing post-thaw motility.

No significant differences were recorded between the resulting mean post-thaw motility rates of the Boran and Nguni bulls, when combining the respective equilibration periods with a 120 minute cooling period. For the Nguni breed equilibration periods of 4 h (33.2±13.4%), 5 h (35.3±11.1%), 8 h (43.1±10.2%), 7 h (43.6±8.6%) and 6 h (44.9±7.4%) yielded increasing mean post-thaw motility rates. For the Boran, for periods of 4 h (31.0±8.9%), 8 h (35.7±3.7%), 7 h (36.0±1.6%), 5 h (36.2±5.4%) and 6 h (37.3±2.8%), increasing mean post-thaw motility rates were recorded.

A 240 minute cooling period yielded no significant differences between the post-thaw sperm motility, when comparing the two breeds at equilibration periods of 4, 6, 7 and 8 h. There was only a tendency towards a difference between the mean post-thaw motility rates for the Boran (35.2±6.8%) and Nguni (44.9±8.0%) breeds, resulting from an equilibration period of 5 h. For the Boran equilibration for periods of 5, 4 (35.8±8.0%), 6 (37.5±2.7%), 8 (38.5±5.8%) and 7 (39.2±3.4%) h yielded increasing post-thaw sperm motility. For the Nguni, periods of 4 (42.4±9.3%), 8 (43.9±9.7%), 7 (44.4±6.5%), 6 (44.8±7.6%) and 5 h yielded increasing post-thaw motility rates.

TABLE 4.9: The between and within breed effect of different glycerol equilibration periods compared with several cooling periods on the mean (±SD) post-thaw sperm motility of bovine semen

COOLING PERIOD (min)	BREED	GLYCEROL EQUILIBRATION PERIOD (h) 4	5	6	7	8
30	B	17.8^{aA} ±13.4	20.7^{abA} ±15.6	28.7^{aBb} ±12.8	28.5^{aBb} ±14.4	33.0^{bB} ±11.9
	N	27.2^{a} ±15.8	25.4^{a} ±14.5	33.1^{ab} ±13.8	32.6^{ab} ±13.1	40.2^{b} ±11.1
60	B	20.2^{a} ±15.1	30.0^{ab} ±11.9	33.2^{b} ±7.0	33.2^{b} ±7.0	34.7^{b} ±4.6
	N	22.8^{a} ±13.6	36.7^{b} ±13.6	35.7^{b} ±16.5	39.6^{b} ±9.9	37.3^{b} ±15.0
120	B	31.0^{a} ±8.9	36.2^{a} ±5.4	37.3^{a} ±2.8	36.0^{a} ±1.6	35.7^{a} ±3.7
	N	33.2^{aA} ±13.4	35.3^{abA} ±11.1	44.9^{bB} ±7.4	43.6^{b} ±8.6	43.1^{aBb} ±10.2
240	B	35.8^{a} ±8.0	$_{aA}35.2^{a}$ ±6.8	37.5^{a} ±2.7	39.2^{a} ±3.4	38.5^{a} ±5.8
	N	42.4^{a} ±9.3	$_{aB}44.9^{a}$ ±8.0	44.8^{a} ±7.6	44.4^{a} ±6.5	43.9^{a} ±9.7
Overall mean (±SD)	**B**	**$_{aA}26.2^{a}$±13.3**	**$_{aA}30.5^{ab}$±11.8**	**$_{aA}34.2^{b}$±8.0**	**$_{a}34.3^{b}$±9.8**	**$_{aA}35.5^{b}$±7.1**
	N	**$_{aB}31.4^{a}$±14.7**	**$_{aB}35.6^{abA}$±13.5**	**$_{aB}39.6^{bc}$±12.7**	**$_{b}40.0^{bBc}$±10.5**	**$_{aB}41.1^{c}$±11.5**

a,b,c Means in the same row with different superscripts differ significantly (p<0.05)

a, b Means in the same cooling and equilibration period with different subscripts differ significantly (p<0.05)

aA.aB Means in the same row with different superscripts tended to differ (p<0.1)

aA,aB Means in the same cooling and equilibration period with different subscripts tended to differ (p<0.1)

a,b,c Means in the same column with different superscripts differ (p<0.05)

aA.aB Means in the same column with different superscripts tended to differ (p<0.1)

B Boran

N Nguni

Figure 4.13: The relationship between glycerol equilibration period and the average post-thaw motility rate of cryopreserved semen from Boran bulls

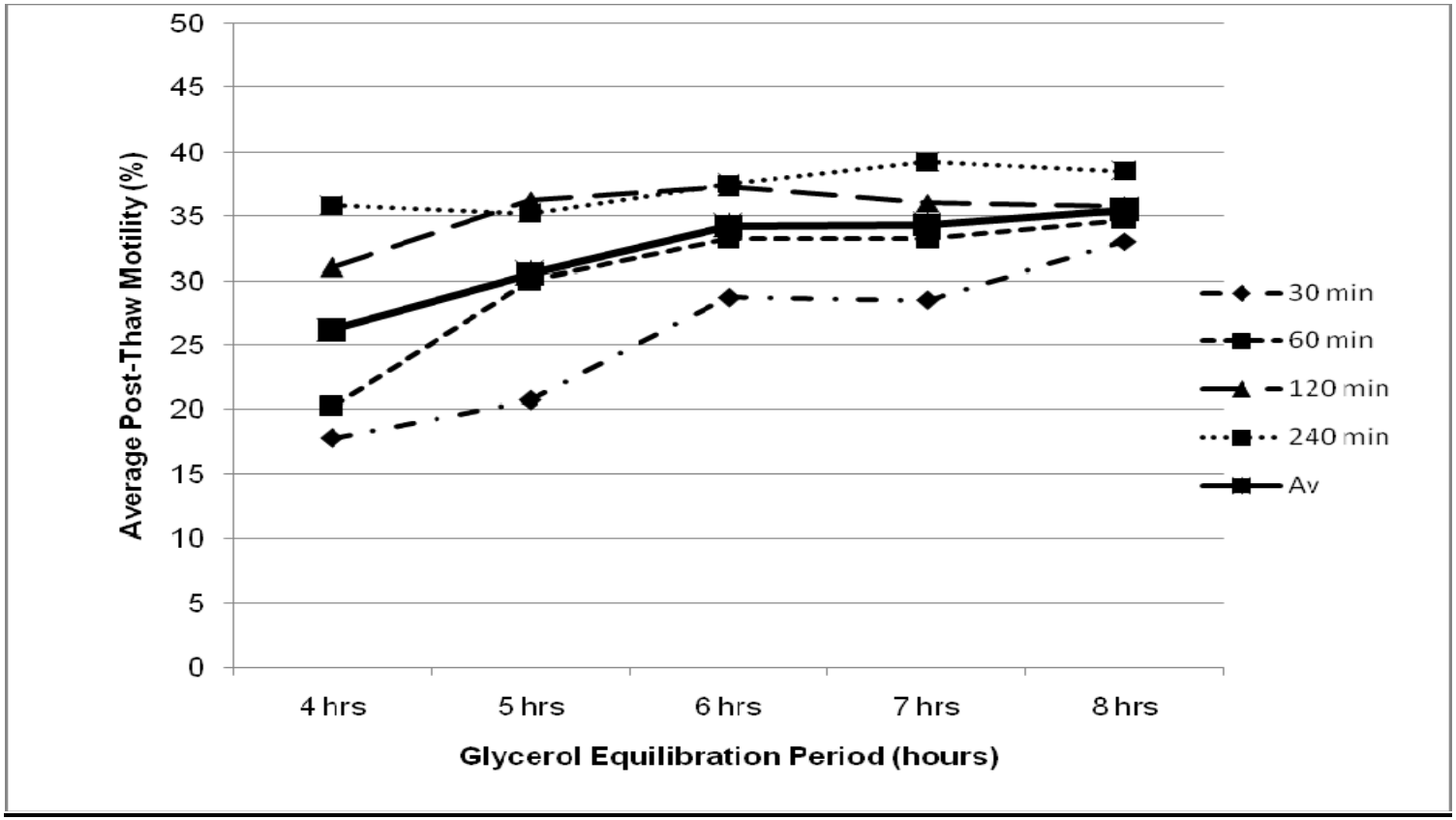
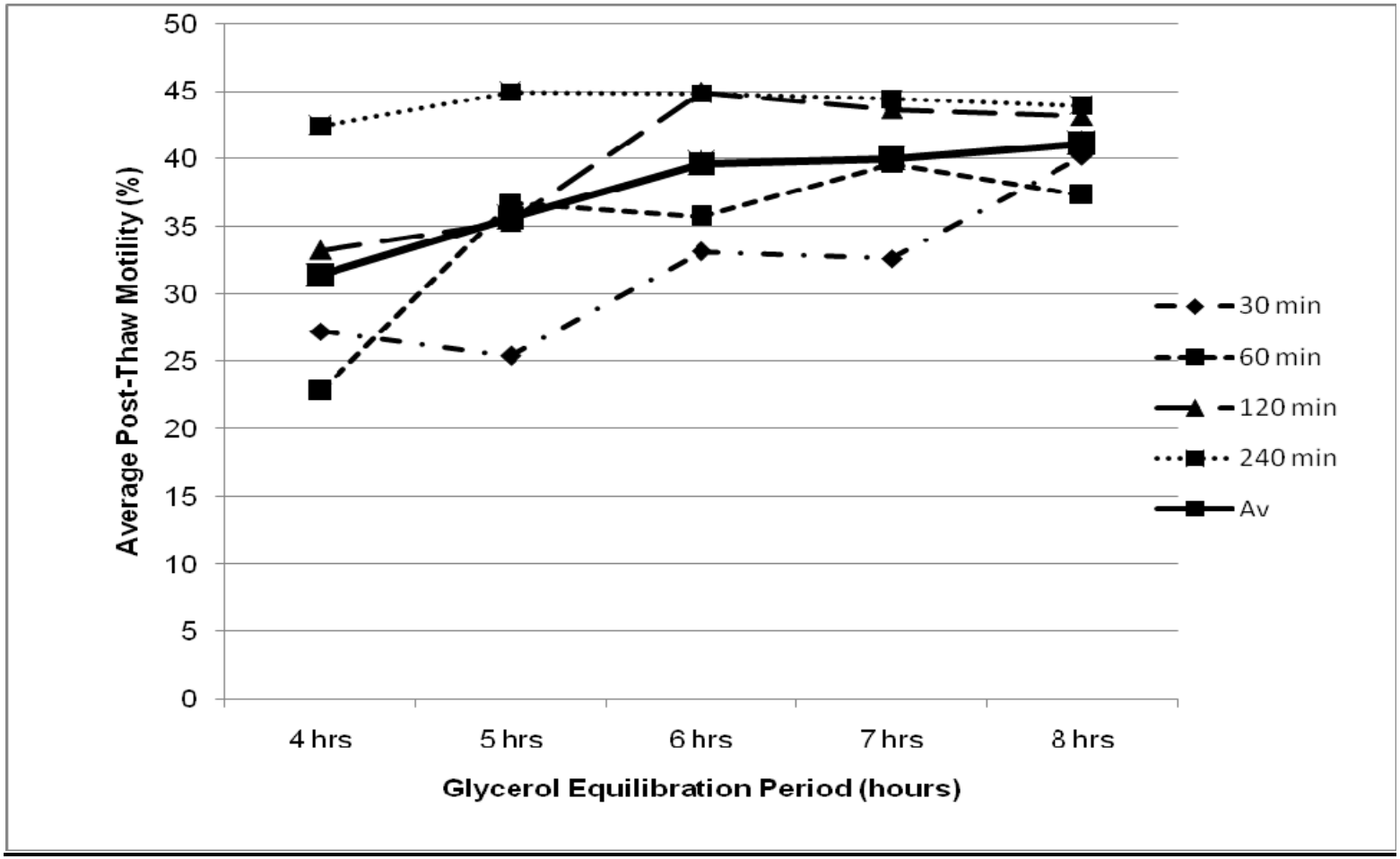


Figure 4.14: The relationship between glycerol equilibration period and mean post-thaw sperm motility rate of cryopreserved semen from Nguni bulls



4.10. Effect of different glycerol equilibration periods on the recovery fraction of Boran and Nguni semen

The effects are summarized in Table 4.10. The general trend for the effect of different glycerol equilibration periods on mean recovery fraction of Boran semen is illustrated in Figure 4.15, and for the Nguni in Figure 4.16.

The author could not find any previous studies using mean recovery fraction of bovine semen as a variable and comparing frozen-thawed semen from Boran and Nguni bulls.

4.10.1. Effect of different glycerol equilibration periods on the recovery fraction of Boran semen

When equilibration periods were compared over all the cooling periods, the mean recovery fractions increased with longer periods of equilibration. Mean recovery fractions of 0.32±0.16, 0.37±0.15, 0.41±0.10, 0.41±0.12 and 0.43±0.09 were recorded after equilibrating semen for 4, 5, 6, 7 and 8 h respectively. There were significant differences ($p<0.05$) recorded between the recovery fractions resulting from 4 and 6, 4 and 7, as well as 4 and 8 h of equilibration. The mean recovery fractions resulting from 5, 6, 7 and 8 h of equilibration were not significantly different from one another. The difference between the mean recovery fractions after 4 and 5 h equilibration were also not significantly different.

With a cooling period of 30 minutes, a glycerol equilibration period of 4 h resulted in the lowest ($p<0.05$; for 8 h and tended to differ from 6 and 7 h) mean recovery fraction (0.22±0.17) while a period of 8 h gave the highest ($p<0.05$; from 4 and 5 h) mean recovery fraction (0.40±0.15). Periods of 5, 7 and 6 h resulted in increasing recovery fractions of 0.25±0.19, 0.35±0.18 and 0.35±0.16 respectively. Equilibration for 4 or 5 h both yielded a significantly lower ($p<0.05$) mean recovery fraction, when compared to the recovery fraction after 8 h. The differences between mean recovery fractions for glycerol equilibration periods of 4 and 8, as well as 5 and 8 h were significant ($p<0.05$).The Boran's mean recovery fractions tended to differ after 4 and 6, as well as after 4 and 7 h. Mean recovery fractions from glycerol equilibration

periods of 6, 7 and 8 h, as well as 5 and 6 and also 5 and 7 h were not significantly different from one another.

With the cooling period of 60 minutes, increasing periods of equilibration resulted in increasing recovery fractions. Equilibration periods of 4, 5, 6, 7 and 8 h yielded recovery fractions of 0.25±0.19, 0.37±0.15, 0.40±0.09, 0.41±0.15 and 0.42±0.06, respectively. The difference between the mean recovery fraction after 4 h of glycerol equilibration was significantly lower ($p<0.05$) than after 6, 7 and 8 h of equilibration. When comparing the mean recovery fractions after 4 and 5 h of equilibration; these means only tended to differ. The mean recovery fractions resulting from glycerol equilibration periods of 5, 6, 7 and h, were not significantly different.

With cooling periods of 120 or 240 minutes, the Boran breed recorded no significant differences between mean recovery fractions from the different glycerol equilibration periods. The mean recovery fractions escalated as the equilibration periods of 4 (0.38±0.11), 8 (0.43±0.05), 7 (0.43±0.03), 5 (0.44±0.08) and 6 h (0.45±0.05) were used in combination with a 120 minute cooling period. When a cooling period of 240 minutes was used, 5 (0.42±0.09), 4 (0.43±0.10), 6 (0.45±0.05), 8 (0.46±0.08) and 7 h (0.47±0.05) of glycerol equilibration resulted in increasing mean recovery fractions.

4.10.2. Effect of different glycerol equilibration periods on the recovery fractions of Nguni semen

For all the cooling periods, there were significant differences ($p<0.05$) regarding the recovery fractions for periods of 4 and 6, 4 and 7, as well as 4 and 8 h. The mean recovery fractions from equilibration periods of 5 and 7, as well as 4 and 5 h differed slightly. Mean recovery fractions after 6, 7 and 8 h of equilibration were not significantly different from one another. Equilibrating semen for a period of either 5 or 6 h yielded recovery fractions that were also not significantly different. The resulting mean recovery fractions after equilibration periods of 4, 5, 6, 7 and 8 h were 0.36±0.16, 0.41±0.15, 0.46±0.14, 0.46±0.11 and 0.47±0.12 respectively.

With a cooling period of 30 minutes, a glycerol equilibration period of 4 h resulted in a significantly lower ($p<0.05$) mean recovery fraction (0.31±0.18) when compared to

8 h (0.46±0.11). For the Nguni breed glycerol equilibration periods of 5 and 7 h also yielded recovery fractions (0.29±0.16 and 0.38±0.14) that differed significantly ($p<0.05$) from the mean recovery fraction after 8 h equilibration (0.46±0.11). The lowest ($p<0.05$; from 8 h) recovery fraction (0.29±0.16) was the result of a 5 h glycerol equilibration period – with increasing fractions for periods of 4 h (0.31±0.18), 7 h (0.38±0.14), 6 h (0.38±0.15) and 8 h (0.46±0.11). There were no significant differences between the mean recovery fractions recorded by glycerol equilibration periods of 4, 5, 6 and 7 h, as well as between the mean recovery fractions for 6 and 8 h.

When a 60 minute cooling period was used, the lowest ($p<0.05$) mean recovery fraction (0.26±0.15) was the result of a 4 h glycerol equilibration, followed by the recovery fraction after 6 h (0.41±0.18). An equilibration period of 5 h yielded the third lowest ($p<0.05$; from 4 h) recovery fraction (0.42±0.14). The highest ($p<0.05$; from 4 h) recovery fraction (0.46±0.09) was the result of a 7 h equilibration period, while a 8 h period resulted in the second highest ($p<0.05$; from 4 h) recovery fraction (0.43±0.16). After 60 minutes of cooling and 4 h of glycerol equilibration the mean recovery fraction differed significantly ($p<0.05$) from that after 5, 6, 7 and 8 h of equilibration.

With a cooling period of 120 minutes, there were significant differences ($p<0.05$) for the Nguni bulls, when comparing recovery fractions following 4 and 6, as well as 4 and 7 h of glycerol equilibration. The recovery fractions after 4 and 8, as well as 5 and 6 h equilibration only tended to differ for the Nguni breed. The lowest values ($p<0.05$; from 6 and 7 h and tended to differ from 8 h) (0.38±0.14) and highest (differed ($p<0.05$) from 4 h) (0.52±0.08) recovery fractions were the result of 4 and 6 h of equilibration. Glycerol equilibration periods of 5, 8 and 7 h resulted in recovery fractions of 0.41±0.12, 0.50±0.10 and 0.50±0.08 respectively. The mean recovery fractions from glycerol equilibration periods of 6, 7 and 8 h, as well as 5 and 7 and 5 and 8 h, were not significantly different from one another.

There were no significant differences between the mean recovery fractions for Nguni bulls, when a cooling period of 240 minutes was used. When equilibration periods of 4 (0.49±0.09), 8 (0.51±0.11), 7 (0.52±0.07), 6 (0.52±0.07) and 5 h (0.52±0.09) were used, the result was increasing 9recovery fractions.

4.10.3. Effects of different glycerol equilibration periods and breed on the recovery fractions of bovine semen

When comparing the mean recovery fractions from the different glycerol equilibration periods over all the cooling periods; there were no significant breed differences. The Boran recorded mean recovery fractions of 0.32±0.16, 0.37±0.15, 0.41±0.10, 0.41±0.12 and 0.43±0.09 after equilibration periods of 4, 5, 6, 7 and 8 h. The Nguni recorded recovery fractions of 0.36±0.16, 0.41±0.15, 0.46±0.14, 0.46±0.11 and 0.47±0.12 after the same periods of equilibration.

When cooling partly diluted semen for 30 minutes before glycerol addition, no significant differences between the recovery fractions of the two breeds after any of the equilibration periods were recorded. The Nguni bulls again recorded consistently higher mean recovery fractions – 0.31±0.18, 0.29±0.16, 0.38±0.15, 0.38±0.14 and 0.46±0.11 - after equilibration periods of 4, 5, 6, 7 and 8 h, respectively, compared to the Boran's recovery fractions of 0.22±0.17, 0.25±0.19, 0.35±0.16, 0.35±0.18 and 0.40±0.15 after the same periods of equilibration.

When a cooling period of 60 minutes was applied, prior to the different equilibration periods, there were no significant differences between the recovery fractions of the two breeds, at any of the equilibration periods. Equilibration periods of 4, 5, 6, 7 and 8 h resulted in mean recovery fractions of 0.25±0.19, 0.37±0.15, 0.40±0.09, 0.41±0.15 and 0.42±0.06 for the Boran and 0.26±0.15, 0.42±0.14, 0.41±0.18, 0.46±0.09 and 0.43±0.16 for the Nguni breed, respectively.

Following a 120 minute cooling period, recovery fractions of semen subjected to equilibration periods of 4, 5, 6, 7 or 8 h showed no significant differences between the breeds. After equilibration periods of 4, 5 and 6 h, recovery fractions of 0.38±0.11, 0.44±0.08 and 0.45±0.05 (Boran) and 0.38±0.14, 0.41±0.12 and 0.52±0.084 (Nguni) were recorded respectively. When diluted semen were allowed to equilibrate for either 7 or 8 h, the mean recovery fractions were 0.43±0.03 and 0.43±0.05 for the Boran, while the Nguni recorded fractions of 0.50±0.08 and 0.50±0.10.

After a cooling period of 240 minutes, there were no significant differences between the recovery fractions for the two breeds, when equilibration periods of 4, 5, 6, 7 and

8 h were applied. The mean recovery fractions after 4 h of equilibration were 0.43±0.10 for the Boran and 0.49±0.09 for the Nguni, respectively. Equilibration for 5 h yielded recovery fractions of 0.42±0.09 (Boran) and 0.52±0.09 (Nguni), while an equilibration period of 6 h resulted in recovery fractions of 0.45±0.05 (Boran) and 0.52±0.07 (Nguni). The recovery fractions of 0.47±0.05 (Boran) and 0.52±0.07 (Nguni) were the result of a 7 h equilibration while a mean recovery fraction of 0.46±0.08 (Boran) and 0.51±0.11 (Nguni) were recorded after 8 h of equilibration.

TABLE 4.10: The between and within breed effect of different glycerol equilibration periods compared within several cooling periods on the mean (±SD) recovery fraction of bovine spermatozoa

COOLING PERIOD	BREED	GLYCEROL EQUILIBRATION PERIOD				
		4	**5**	**6**	**7**	**8**
30	B	0.22^{aA} ±0.17	0.25^{a} ±0.19	0.35^{aBb} ±0.16	0.35^{aBb} ±0.18	0.40^{b}±0.15
	N	0.31^{a} ±0.18	0.29^{a} ±0.16	0.38^{ab} ±0.15	0.38^{a} ±0.14	0.46^{b} ±0.11
60	B	0.25^{aA} ±0.19	0.37^{aBb} ±0.15	0.40^{b} ±0.09	0.41^{b} ±0.15	0.42^{b} ±0.056
	N	0.26^{a} ±0.15	0.42^{b} ±0.14	0.41^{b} ±0.18	0.46^{b} ±0.09	0.43^{b} ±0.16
120	B	0.38^{a} ±0.11	0.44^{a} ±0.08	0.45^{a} ±0.05	0.43^{a} ±0.03	0.43^{a} ±0.05
	N	0.38^{aA} ±0.14	0.41^{abA} ±0.12	0.52^{bB} ±0.08	0.50^{b} ±0.08	$0.50^{aB,b}$ ±0.10
240	B	0.43^{a} ±0.10	0.42^{a} ±0.09	0.45^{a} ±0.05	0.47^{a} ±0.05	0.46^{a} ±0.08
	N	0.49^{a} ±0.09	0.52^{a} ±0.09	0.52^{a} ±0.07	0.52^{a} ±0.07	0.51^{a} ±0.11
Overall Mean (±SD)	B	**$_{a}0.32^{a}$±0.16**	**$_{a}0.37^{ab}$±0.15**	**$_{a}0.41^{b}$±0.10**	**$_{a}0.41^{b}$±0.12**	**$_{a}0.43^{b}$±0.09**
	N	**$_{a}0.36^{aA}$±0.16**	**$_{a}0.41^{aBbA}$±0.15**	**$_{a}0.46^{bc}$±0.14**	**$_{a}0.46^{bBc}$±0.11**	**$_{a}0.47^{c}$±0.12**

^{a,b,c} Means in the same row with different superscripts differ significantly ($p<0.05$)
$_{a,\ b}$ Means in the same cooling and equilibration period with different subscripts differ significantly ($p<0.05$)
$^{aA.aB}$ Means in the same row with different superscripts tended to differ ($p<0.1$)
$_{aA,aB}$ Means in the same cooling and equilibration period with different subscripts tended to differ ($p<0.1$)
a,b,c Means in the same column with different superscripts differ ($p<0.05$)
aA.aB Means in the same column with different superscripts tended to differ ($p<0.1$)
B Boran
N Nguni

Figure 4.15: The relationship between the glycerol equilibration period and mean recovery fraction of cryopreserved semen from Boran bulls

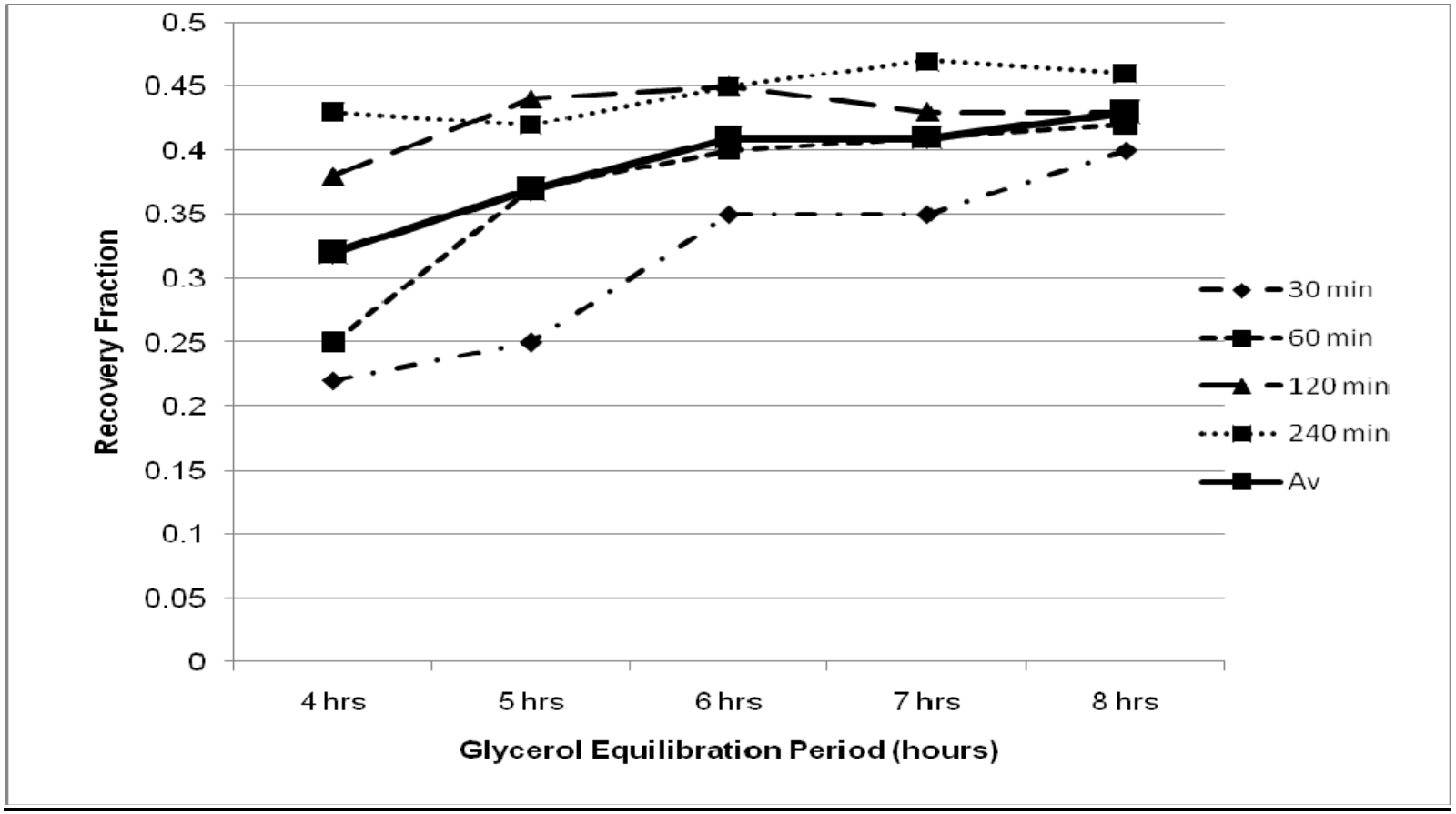
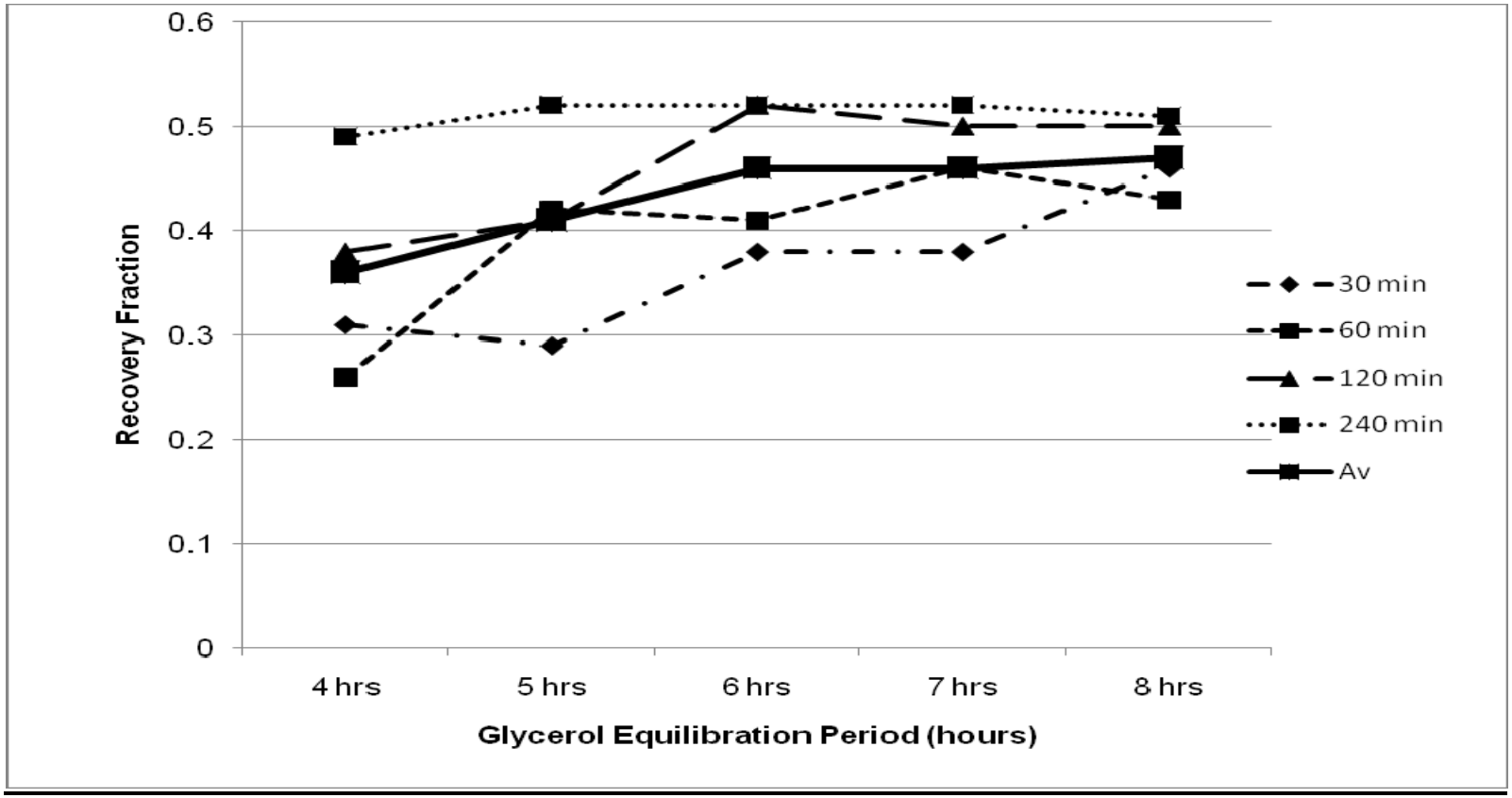


Figure 4.16: The relationship between the glycerol equilibration period and mean recovery fraction of cryopreserved semen from Nguni bulls



From a practical perspective, a user-friendly matrix comparing the different combinations of cooling and glycerol equilibration periods studied in Experiment 1 would be of value to the semen cryopreservation industry. This cooling period and glycerol equilibration period matrix would be a helpful tool in managing the quality of cryopreserved semen from these African indigenous bulls.

4.11. Effect of different combinations of cooling and glycerol equilibration periods on the post-thaw sperm motility of bovine semen

The mean post-thaw motility for cooling periods over all the glycerol equilibration periods increased as the period of cooling increased. A 30 minute cooling period resulted in a mean post-thaw motility of 28.7±14.3%, while cooling periods of 60, 120 and 240 minutes yielded mean post-thaw motility rates of 32.4±13.5%, 37.6±9.4% and 40.7±7.8%, respectively. The post-thaw motility rate after a 30 minute cooling period was significantly lower ($p<0.05$) than the post-thaw motility after any of the longer cooling periods. A cooling period of 60 minutes resulted in a mean post-thaw motility rate that was significantly lower ($p<0.05$) than that after either 120 or 240 minutes of cooling. The mean post-thaw motility after 120 or 240 minutes of cooling, also differed significantly ($p<0.1$) from one another.

Glycerol equilibration periods of 4, 5, 6, 7 and 8 h resulted in an increasing mean post-thaw motility of 28.8±14.3%, 33.0±13.0%, 36.9±11.3%, 37.2±10.6% and 38.3±10.3%. The mean post-thaw motility rate after 4 h of glycerol equilibration was lowest, and significantly differed ($p<0.05$) from the mean post-thaw motility after all the longer equilibration periods. The mean post-thaw motility after 5 or 6 h of glycerol equilibration only tended to differ significantly from each other, while the mean post-thaw motility after 7 or 8 h of glycerol equilibration was significant ($p<0.05$). Glycerol equilibration periods of either 7 or 8 h yielded mean post-thaw motility rates that did not differ significantly.

The lowest mean rate of post-thaw sperm motility (21.5±13.7%) was the result of combining a 60 minute cooling period and a 4 h glycerol equilibration period. This post-thaw motility did not differ significantly from cooling period-glycerol equilibration period combinations of 30 minutes cooling followed by glycerol equilibration periods of 4, 5, 6 and 7 h which yielded the second, third and fourth lowest post-thaw motility

rates of 22.5±15.1%, 23.1±14.6% and 30.5±13.3% respectively. The mean post-thaw motility resulting from a combination of 60 minutes cooling and 4 h glycerol equilibration did however differ significantly ($p<0.05$) from the mean post-thaw motility rates – resulting from all the other combinations of cooling and glycerol equilibration periods.

The second lowest mean post-thaw sperm motility (22.5±15.1%), yielded by the combination of 30 minutes cooling and 4 h glycerol equilibration, tended to differ significantly from the mean post-thaw motility rate after 30 minutes of cooling and 7 h glycerol equilibration, as well as at $p<0.05$ from combining 30 minutes of cooling with 6 or 8 h glycerol equilibration, or 60 minutes of cooling with 5, 6, 7 or 8 h of glycerol equilibration, or 120 or 240 minutes of cooling with any of the 5 glycerol equilibration periods.

The third lowest post-thaw motility (23.1±14.6%) was the result of combining 30 minutes of cooling with 5 h of glycerol equilibration. This rate was significantly lower ($p<0.05$) than the mean post-thaw motility rates after any combinations with a cooling period, of either 120 or 240 minutes, as well as combinations of 30 minutes cooling with 8 h glycerol equilibration, or 60 minutes of cooling with 5, 6, 7 or 8 h of glycerol equilibration. The third lowest post-thaw motility also tended to be significantly lower than the mean post-thaw motility rate, after combining a 30 minute cooling period with either 6 or 7 h of glycerol equilibration.

The mean post-thaw sperm motility rates after a cooling period of 30 minutes and either 6 or 7 h of glycerol equilibration were 30.9±13.1% and 30.5±13.3%, respectively. These mean post-thaw motility rates were significantly lower than any of the combinations with 240 minutes of cooling, as well as combinations of 120 minutes cooling with 6, 7 or 8 h of glycerol equilibration.

Combining a cooling period of 120 minutes with a glycerol equilibration period of 4 h yielded a mean post-thaw motility rate of 32.1±11.5%. This rate was significantly lower ($p<0.05$), than post-thaw motility rates, resulting from a cooling period of 120 minutes – followed by either 6 or 8 h of equilibration, or a cooling period of 240 minutes followed by a glycerol equilibration period of 6, 7 or 8 h. The mean post-thaw sperm motility rate after a cooling period of 120 minutes, combined with a

glycerol equilibration period of 4 h also differed significantly from mean post-thaw motility rates after cooling for 120 minutes – and a 7 h glycerol equilibration period or cooling for 240 minutes, followed by a glycerol equilibration period of either 4 or 5 h.

A cooling period of 60 minutes followed by a 5 h glycerol equilibration period resulted in a mean post-thaw motility rate of 33.3±12.9%. This mean was significantly lower ($p<0.05$) than the post-thaw motility rate yielded by the combination of a 240 minute cooling period and a 7 h glycerol equilibration period. The mean post-thaw sperm motility rate from the 60 minute-5 h cooling period glycerol equilibration period combination also tended to be significantly lower than the mean post-thaw motility resulting from a cooling period of 120 minutes combined with a 6 h glycerol equilibration period - or a cooling period of 240 minutes, combined with either 6 or 8 h of glycerol equilibration.

The 60 minute cooling and 6 h glycerol equilibration period combination recorded post-thaw sperm motility (34.4±13.2%) that tended to differ from the results of a cooling period of 240 minutes combined with a glycerol equilibration period of either 7 or 8 h.

Combinations of 30 minutes-8 h, 60 minutes-7 or 8 h, 120 minutes-5, 6, 7 or 8 h and 240 minutes-4, 5, 6, 7 or 8 h equilibration yielded mean post-thaw sperm motility rates - 36.6±11.6%, 36.5±10.9%, 36.0±11.8%, 35.8±9.0%, 41.1±7.0%, 39.8±7.6%, 39.4±8.8%, 39.1±9.1%, 40.0±8.8%, 41.1±7.0%, 41.8±6.0% and 41.2±8.6%, respectively – that did not differ significantly.

TABLE 4.11: The overall effect of different combinations of cooling and glycerol equilibration periods on the mean (±SD) post-thaw sperm motility rate of bovine semen

GLYCEROL EQUILIBRATION PERIOD (h)	COOLING PERIOD (minutes) 30	60	120	240	Mean
4	22.5^{aA}±15.1	21.5^{a}±13.7	32.1^{cgA}±11.5	39.1^{defgB}±9.1	**28.8^{a}±14.3**
5	23.1^{aAbA}±14.6	33.3^{ceA}±12.9	35.8^{cef}±9.0	40.0^{defgB}±8.8	**33.0^{bA}±13.0**
6	30.9^{bBc}±13.1	34.4^{cfA}±13.2	41.1^{deBf}±7.0	41.1^{deBf}±7.0	**36.9^{bB}±11.3**
7	30.5^{aBc}±13.3	36.5^{cf}±10.9	39.8^{defgB}±7.6	41.8^{dfB}±6.0	**37.2^{c}±10.6**
8	36.6^{cd}±11.6	36.0^{cef}±11.8	39.4^{def}±8.8	41.2^{deBfB}±8.6	**38.3^{c}±10.3**
Overall mean (±SD)	**28.7^{a}±14.3**	**32.4^{b}±13.5**	**37.6^{cA}±9.4**	**40.7^{cB}±7.8**	

^{a,b,c} Significant difference ($p<0.05$)

$^{\underline{a,b,c}}$ Significant difference ($p<0.05$) between mean values

$^{A.B}$ Inclination to significant difference from other values with the same ^{a,b,c} symbol ($p<0.1$)

$^{\underline{A.B}}$ Inclination to significant difference from other values with the same $^{\underline{a,b,c}}$ symbol ($p<0.1$)

The results obtained in Experiment 1 suggest that the optimum glycerol equilibration period used in the cryopreservation process of indigenous African bulls are dependent on the cooling period to which semen are subjected prior to glycerol addition. Because of this observation, a second experiment was conducted to further study the interactions between the cooling period and glycerol equilibration periods were conducted.

CHAPTER 5

RESULTS AND DISCUSSION FOR EXPERIMENT 2

5.1. Effect of total equilibration periods on the post-thaw sperm motility of bovine semen

The results regarding equilibration period and post-thaw sperm motility are summarized in Table 5.1.

Semen samples equilibrated for the shortest total equilibration period (4.5 h) recorded a mean post-thaw motility of 31.00±13.5%. Increasing the total equilibration period of the semen samples to either 5 or 5.5 h resulted in slightly higher though non-significant higher post-thaw motility rates of 34.30±10.6% and 40.00±10.7% respectively. The mean post-thaw sperm motility resulting from total equilibration periods of 4.5 and 5 h as well as 5 and 5.5 h were not significantly different. Total equilibration periods of 4.5 and 5.5 h, however recorded post-thaw motility rates that differed significantly ($p<0.05$).

When applying total equilibration periods of 6, 6.5, 7, 7.5, 8 or 8.5 h to several semen samples, the resulting mean post-thaw motility rates were 38.25±9.2%, 39.10±8.8%, 42.58±10.1%, 41.30±10.8%, 41.30±8.1% or 43.30±12.0% for the respective periods. The resulting mean post-thaw sperm motility after 4.5 h for the total equilibration period differed significantly ($p<0.05$) from all other total equilibration periods, except the 5 h equilibration period. The 5 h period yielded an mean post-thaw motility that was significantly lower ($p<0.05$) than the post-thaw motility after periods of 7, 8 and 8.5 h and also tended to differ ($p<0.1$) from mean post-thaw sperm motility rate recorded by a total equilibration period of 7.5 h. Mean post-thaw motility resulting from 5.5, 6. 6.5, 7, 7.5, 8 or 8.5 h of total equilibration were not significantly different from one another.

Equilibrating semen samples for longer periods (9, 10, 11 or 12 h) resulted in the higher – although not significantly different, post-thaw motility rates. Periods of 9, 10, 11 and 12 h yielded a mean post-thaw sperm motility rates of 42.4±8.7%, 42.3±9.2%,

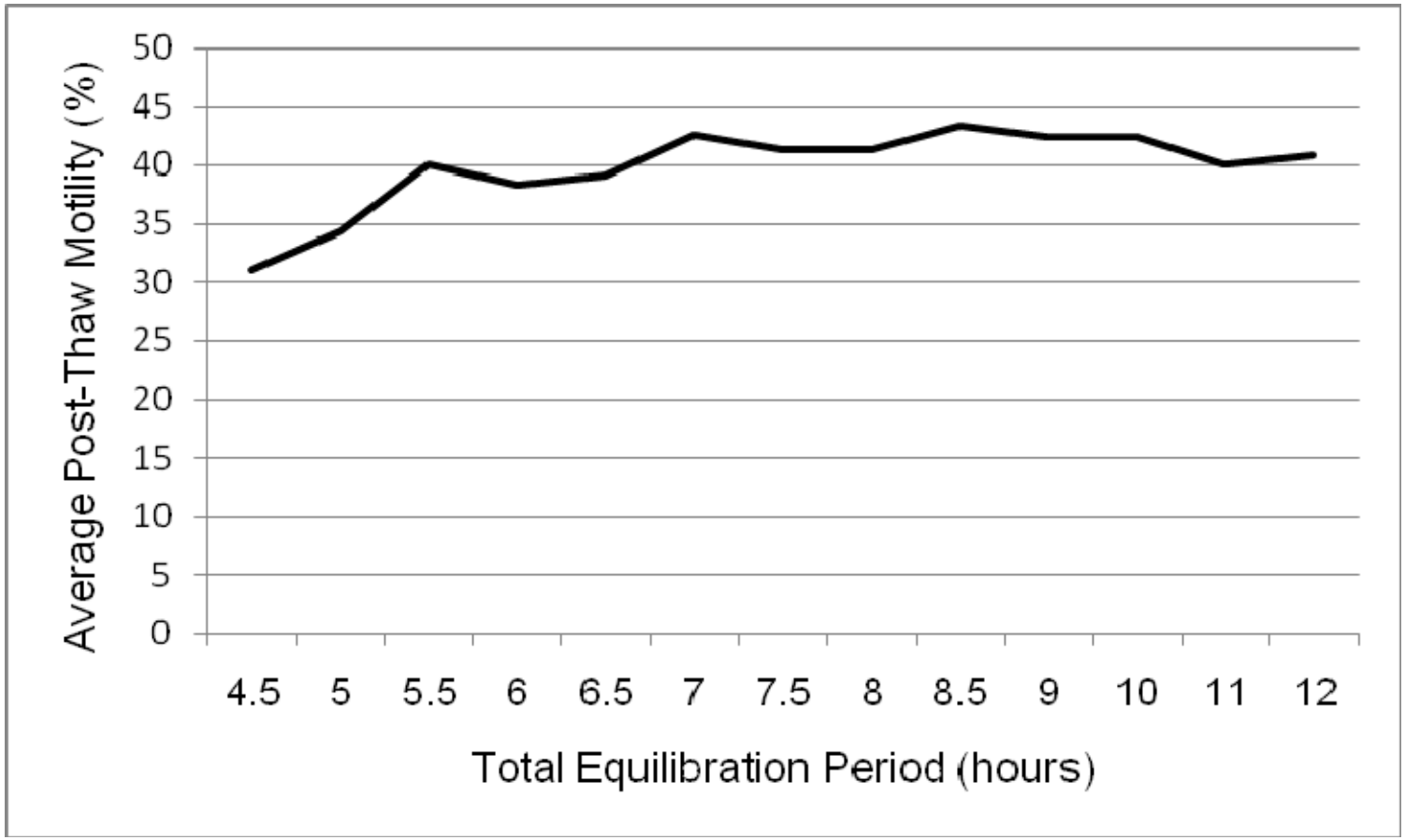
40.0±13.3% and 40.7±8.1% respectively. These post-thaw motility rates were not significantly different from one another, or from the mean post-thaw motility yielded by 5.5, 6, 6.5, 7, 7.5, 8 or 8.5 h of equilibration, but were all significantly higher ($p<0.05$) than the post-thaw motility rates after a total equilibration periods of 4.5 h. Mean post-thaw motility rates resulting from 9 and 10 h of equilibration also differed significantly ($p<0.0.5$) from the mean after 5 h of equilibration.

TABLE 5.1: Effects of different total equilibration periods on the mean (±SD) post-thaw sperm motility of bovine semen

Total Equilibration Time (h)	Mean (±SD) Post-Thaw Sperm Motility (%)
4.5	$31.0^{a} \pm 13.5$
5	$34.3^{abA} \pm 10.6$
5.5	$40.0^{bc} \pm 10.7$
6	$38.3^{bc} \pm 9.2$
6.5	$39.1^{bc} \pm 8.8$
7	$42.6^{c} \pm 10.1$
7.5	$41.3^{bBc} \pm 10.8$
8	$41.3^{c} \pm 8.1$
8.5	$43.3^{c} \pm 12.0$
9	$42.4^{c} \pm 8.7$
10	$42.3^{c} \pm 9.2$
11	$40.0^{bc} \pm 13.3$
12	$40.7^{bc} \pm 8.1$

^{a,b,c} Means with different superscript letters differ ($p<0.05$)
$^{A.B}$ Means with different superscript letter tended to differ ($p<0.1$)

Figure 5.1: The relationship between total equilibration period and mean post-thaw sperm motility rate of cryopreserved bovine semen



5.2. Effect of equilibration periods on the recovery fraction in bovine semen

The results following equilibration and recovery fraction are summarized in Table 5.2. The general trend regarding the effect of total equilibration period on mean recovery fraction of semen is also illustrated in Figure 5.2. The author could not find any previous studies, using mean recovery fraction of bovine semen as a variable.

Semen samples equilibrated for a total equilibration period of 4.5 h recorded the lowest ($p<0.05$) recovery fraction of 0.36±0.15. Increasing the total equilibration period of the semen samples to either 5 or 5.5 h, resulted in the recovery fractions of 0.40±0.12 and 0.47±0.11 respectively. The mean recovery fractions recorded following total equilibration periods of 4.5 and 5 h, as well as 5 and 5.5 h were not significantly different. Total equilibration periods of 4.5 and 5.5 h, however recorded recovery fractions that significantly differed ($p<0.05$).

When applying total equilibration periods of 6, 6.5, 7, 7.5 or 8 h to several semen samples, the resulting mean recovery fractions were 0.45±0.10, 0.46±0.09, 0.50±0.10, 0.48±0.11 and 0.49±0.09 for the respective periods. The resulting mean recovery fractions after 4.5 h of total equilibration, was significantly lower ($p<0.05$) than all other equilibration periods – except the 5 h equilibration period. The 5 h total equilibration period recorded an mean recovery fraction that was significantly lower ($p<0.05$) than the fractions after equilibration periods of 7 and 8 h, while also tending to differ from the mean recovery fractions recorded following a total equilibration period of 7.5 h. The mean recovery fractions for 6, 6.5, 7, 7.5 or 8 h of equilibration were not significantly different.

Equilibrating semen samples for longer periods (8.5, 9, 10, 11 or 12 h) resulted in the highest ($p<0.05$) recovery fractions. Periods of 8.5, 9, 10, 11 and 12 h recorded mean recovery fractions of 0.51±0.12, 0.50±0.09, 0.50±0.10, 0.47±0.15 and 0.48±0.09, respectively. These recovery fractions were not significantly different from one another, or from the recovery fractions yielded by 6.5, 7, 7.5 or 8 h of equilibration. They were however all significantly higher ($p<0.05$) than the mean recovery fractions after a total equilibration periods of 4.5 h. Mean recovery fractions resulting from total equilibration periods of 7, 8, 9 and 10 h differed significantly ($p<0.05$) from the recovery fraction after 5 h of equilibration. A 5 h total equilibration

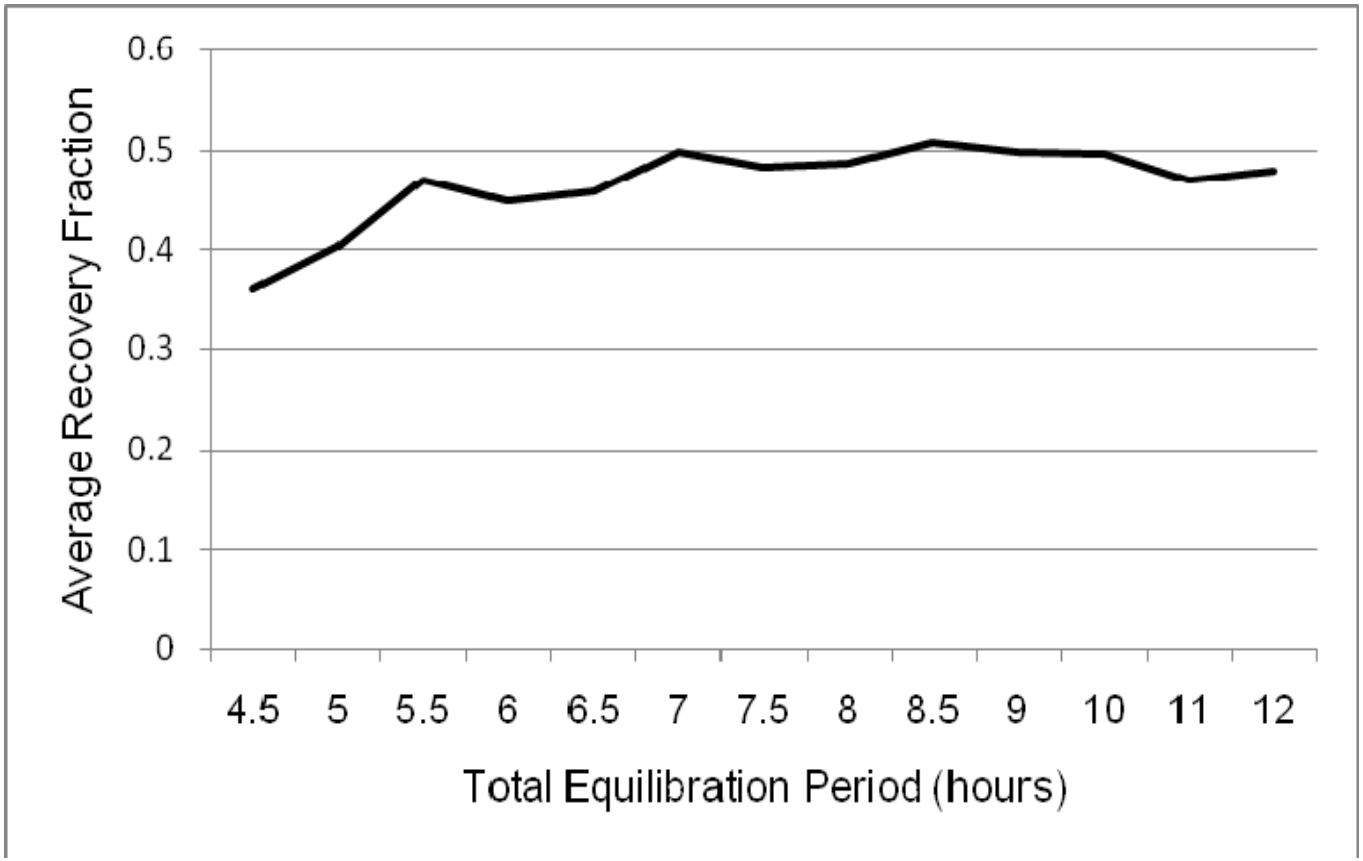
resulted in a recovery fraction that tended to be lower, than the recovery fraction for a total equilibration period of 12 h.

TABLE 5.2: Effect of different total equilibration periods on the mean (±SD) recovery fraction of bovine spermatozoa

Total Equilibration Time (h)	Mean (±SD) Recovery Fraction
4.5	$0.36^{a} \pm 0.15$
5	$0.40^{abA} \pm 0.12$
5.5	$0.47^{bc} \pm 0.11$
6	$0.45^{bcA} \pm 0.10$
6.5	$0.46^{bc} \pm 0.09$
7	$0.50^{c} \pm 0.10$
7.5	$0.48^{bBc} \pm 0.11$
8	$0.49^{c} \pm 0.09$
8.5	$0.51^{c} \pm 0.12$
9	$0.50^{cB} \pm 0.09$
10	$0.50^{c} \pm 0.10$
11	$0.47^{bc} \pm 0.15$
12	$0.48^{bBc} \pm 0.09$

^{a,b,c} = Means with different superscripts differ ($p<0.05$)
$^{A.B}$ = Means with different superscripts tended to differ ($p<0.1$)

Figure 5.2: The relationship between the total equilibration period and mean recovery fractions of cryopreserved bovine semen



5.3. Effect of equilibration periods on the post-thaw sperm motility of semen from Boran and Nguni bulls

The results for the equilibration period and the post-thaw sperm motility are summarized in Table 5.3. The general trend for the effect of total equilibration period on the post-thaw sperm motility for Boran and Nguni semen is also illustrated in Figure 5.3.

The author could not find any studies comparing frozen-thawed semen from Boran and Nguni bulls.

5.3.1. Effect of total equilibration period on the post-thaw sperm motility of Boran semen

When semen samples were equilibrated for the shortest total equilibration period of 4.5 h, the resulting post-thaw motility was 26.0±10.8%. Increasing the total equilibration period of the semen samples to 5 h resulted in a post-thaw motility of 28.0±10.4, thus an increase of 2% in the mean post-thaw sperm motility. However, the post-thaw sperm motility resulting from total equilibration periods of 4.5 and 5 h were not significantly different.

When subjecting semen samples to total equilibration periods of 5.5 6, 6.5, 7, 7.5, 8 or 8.5 h the resulting post-thaw motility rates recorded were 35.8±3.1%, 35.7±2.9%, 35.0±0.0%, 37.4±2.2%, 35.2±4.0%, 37.9±3.9% and 37.0±5.1% for the respective periods. The 4.5 h period yielded a mean post-thaw motility rate that was significantly lower ($p<0.05$) than the mean post-thaw motility rates after periods of 7 and 8 h and also tended to differ from the post-thaw sperm motility recorded for periods of 5.5, 6 and 8.5 h. There were no significant differences between mean post-thaw motility rates of the 4.5, and either the 6.5 or 7.5 h equilibration periods. The post-thaw motility resulting from 5 h of total equilibration were not significantly different from the post-thaw motility recorded for periods of 5.5, 6, 6.5, 7.5 or 8.5 h. However, the mean post-thaw motility rate after 5 h of total equilibration tended to be significantly lower than the mean post-thaw motility rates recorded for 7 or 8 ($p<0.05$)

h of equilibration. Mean post-thaw motility rates resulting from 5.5, 6, 6.5, 7, 7.5, 8 or 8.5 h of total equilibration were not significantly different from one another.

When equilibrating semen samples for longer periods of time (9, 10, 11 or 12 h) resulted in mean post-thaw sperm motility rates of 39.1±3.5%, 37.9±4.0%, 37.0±9.7% and 37.2±5.2%, respectively. The mean post-thaw motility rates mentioned above were not significantly different from one another or from the mean post-thaw motility rates yielded by shorter periods of 5.5, 6, 6.5, 7, 7.5, 8 or 8.5 h of equilibration. The post-thaw motility resulting from 9 or 10 h total equilibration were however, significantly higher ($p<0.05$) than the motility after equilibration periods of either 4.5 or 5 h. Although the mean post-thaw motility rates yielded by equilibration periods of 11 and 12 h both tended to be significantly higher than the motility after an equilibration period of 4.5 h, there was no significant difference between the mean post-thaw sperm motility rates recorded following 11, 12 or 5 h of equilibration.

5.3.2. Effects of total equilibration periods on the post-thaw sperm motility of Nguni semen

The shortest total equilibration periods (4.5, 5 or 5.5 h) applied to semen samples yielded mean post-thaw motility rates of 36.0±15.2%, 40.6±6.7% and 44.2±14.3%. The mean post-thaw motility rates resulting from equilibration periods of 4.5, 5 or 5.5 h were not significantly different from one another.

Equilibrating semen samples for total equilibration periods of 6, 6.5, 7 or 7.5 h, the resulting mean post-thaw motility rates were 40.8±12.4%, 43.2±11.6%, 47.8±11.8% and 47.4±12.5%, for the respective periods. The resulting mean post-thaw motility rate after 4.5 h of equilibration differed significantly ($p<0.05$) from the mean post-thaw motility after total equilibration periods of 7 and 7.5 h, but were not significantly different from the post-thaw motility rates recorded for 6 or 6.5 h of equilibration. The 5 and 5.5 h periods both recorded mean post-thaw sperm motility rates that were not significantly different from the mean motility after periods of 6, 6.5, 7 or 7.5 h. The mean post-thaw motility rates yielded by total equilibration periods of 6, 6.5, 7 or 7.5 h were also not significantly different from one another.

Subjecting semen samples to longer periods of equilibration generally resulted in slightly higher ($p<0.05$) post-thaw motility rates. Periods of 8, 8.5, 9, 10, 11 and 12 h yielded mean post-thaw motility rates of 44.7±9.8%, 49.6±14.1%, 45.7±11.1%, 46.8±11.3%, 43.0±16.8 and 44.2±9.5%, respectively. The above-mentioned post-thaw motility rates were not significantly different from one another, or from mean post-thaw motility rates yielded by equilibration periods of 5, 5.5, 7 or 7.5 h. Total equilibration periods of 8.5, 9 and 10 h all yielded mean post-thaw motility rates that were significantly higher ($p<0.05$), than the mean motility rates after a period of 4.5 h. The mean post-thaw sperm motility rate, resulting from 4.5 h total equilibration, also tended to be significantly lower than the mean post-thaw motility rate resulting from 8 h of equilibration. These were however not significantly different from the mean post-thaw motility yielded by either 11 or 12 h equilibration. Equilibration periods of either 8, 9, 10, 11 or 12 h resulted in mean post-thaw motility rates that were not significantly higher than the mean post-thaw motility rate yielded by a 6 h equilibration period. There was a tendency towards significant difference between the mean post-thaw motility rates resulting from total equilibration periods of either 6 or 8.5 h.

5.3.3. Effect of equilibration periods and breed on the differences in post-thaw sperm motility of bovine semen

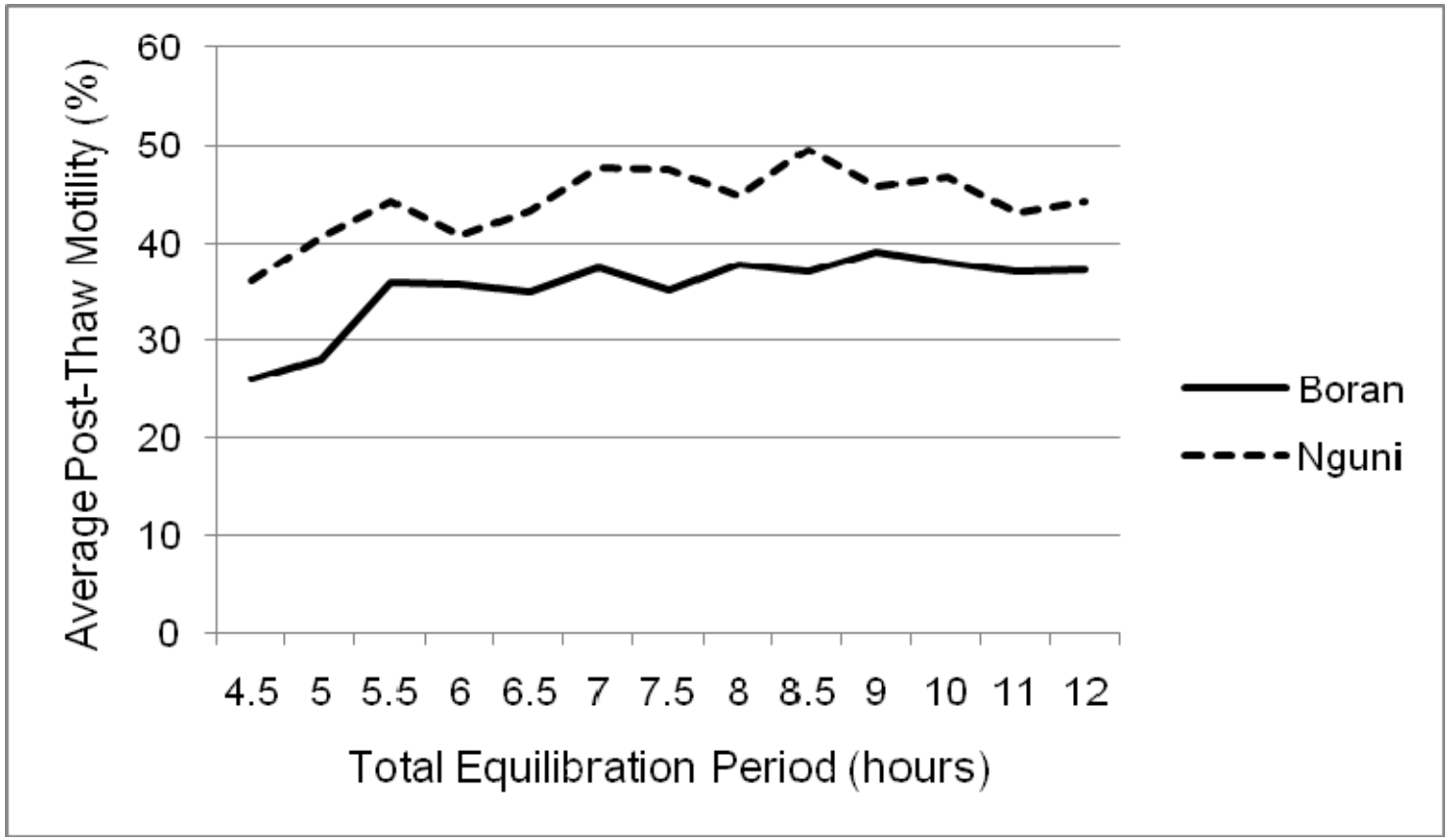
Mean post-thaw motility rates yielded by the two cattle breeds after total equilibration periods of 5, 7, 7.5, 8, 8.5 or 10 h, differed significantly ($p<0.05$), with the Nguni recording the higher post-thaw motility rate. With total equilibration periods of either 4.5 or 9 h, the mean post-thaw motility rate yielded by the Nguni breeds also tended to be higher than the post-thaw motility yielded by the Boran breed. After total equilibration periods of 5.5, 6, 6.5, 11 or 12 h, the difference between the mean post-thaw motility yielded by the Boran and Nguni breeds were not significantly different.

TABLE 5.3: Effects of different equilibration periods on the mean (±SD) post-thaw sperm motility of Boran and Nguni semen

Total Equilibration Time (h)	BREED	
	Boran	**Nguni**
	Mean Post-Thaw Motility (%)	Mean Post-Thaw Motility (%)
4.5	$_{aA}26.0^{aA}$ ± 10.8	$_{aB}36.0^{aA}$ ± 15.2
5	$_{a}28.0^{abA}$ ± 10.4	$_{b}40.6^{ab}$ ± 6.7
5.5	35.8^{aBc} ± 3.1	44.2^{ab} ±14.3
6	35.7^{aBc} ± 2.9	40.8^{abA} ± 12.4
6.5	35.0^{ac} ± 0.0	43.2^{ab} ± 11.6
7	$_{a}37.4^{bBc}$ ± 2.2	$_{b}47.8^{bB}$ ± 11.8
7.5	$_{a}35.2^{ac}$ ± 4.0	$_{b}47.4^{b}$ ± 12.5
8	$_{a}37.9^{c}$ ± 3.9	$_{b}44.7^{aBb}$ ± 9.8
8.5	$_{a}37.0^{aBc}$ ± 5.1	$_{b}49.6^{bB}$ ± 14.1
9	$_{aA}39.1^{c}$ ± 3.5	$_{aB}45.7^{b}$ ± 11.1
10	$_{a}37.9^{c}$ ± 4.0	$_{b}46.8^{b}$ ± 11.3
11	37.0^{aBc} ± 9.7	43.0^{ab} ± 16.8
12	37.2^{aBc} ± 5.2	44.2^{ab} ± 9.5

a,b,c Means in the same column with different superscripts differ significantly ($p<0.05$)
a,b,c Means in the same row with different superscripts differ significantly ($p<0.05$)
A.B Means in the same column with different superscript letter tended to differ ($p<0.1$)
A.B Means in the same row with different superscript letter tended to differ ($p<0.1$)
B Boran bulls
N Nguni bulls

Figure 5.3: The relationship between the total equilibration period and mean post-thaw sperm motility of cryopreserved semen of Boran and Nguni bulls



5.4. Effect of total equilibration periods on the recovery fraction of semen from Boran and Nguni bulls

The results of the effect of equilibration on recovery fraction are summarized in Table 5.4. The general trend regarding the effect of total equilibration period on the mean recovery fraction Boran and Nguni semen is illustrated in Figure 5.4.

The author could not find any previous studies using mean recovery fraction of bovine semen as a variable. The author could not find any previous studies comparing frozen-thawed semen from Boran and Nguni bulls.

5.4.4. Effects of total equilibration period on the recovery fractions of Boran semen

The shortest total equilibration period (4.5 h) applied to Boran semen samples recorded a mean recovery fraction of 0.31±0.13. By increasing the total equilibration period of the semen to 5h, resulted in a slightly higher (although not significantly different) with a mean recovery fraction of 0.34±0.12. The mean recovery fractions resulting from equilibration periods of 4.5 and 5 h did not differ significantly from one another.

When semen samples were allowed to equilibrate for periods of 5.5, 6, 6.5, 7 or 7.5 h the resulting mean recovery fractions were 0.43±0.05, 0.43±0.04, 0.42±0.02, 0.45±0.04 and 0.42±0.05 for the respective periods. The resulting recovery fractions after 6 or 7 h of equilibration were significantly higher ($p<0.05$) than the mean resulting from 4.5 h equilibration. The mean recovery fraction following 4.5 h equilibration also tended to be lower than the recovery fractions from 5.5, 6.5 and 7.5 h periods. A total equilibration period of 5 h yielded a mean recovery fraction that was not significantly different from the recovery fraction resulting from equilibration periods of 5.5, 6.5 or 7.5 h. The mean recovery fraction resulting from a 5 h equilibration period also tended to be lower than the recovery fraction after 6 and 7 h ($p<0.05$) of total equilibration. Mean recovery fractions resulting from 5.5, 6, 6.5, 7 and 7.5 h of total equilibration were not significantly different from one another.

Equilibrating semen samples for longer periods (8, 8.5, 9, 10, 11 or 12 h) resulted in the mean recovery fractions of 0.46±0.05, 0.45±0.06, 0.47±0.05, 0.46±0.06, 0.45±0.12 and 0.45±0.07, respectively. The above-mentioned recovery fractions were not significantly different from one another, or from mean recovery fractions resulting from the shorter equilibration periods of 5.5, 6, 6.5, 7 or 7.5 h. The recovery fractions after equilibration periods of 4.5 or 5 h were however significantly lower ($p<0.05$) than the recovery fractions yielded by total equilibration periods of 8, 9 or 10 h. Total equilibration periods of 8.5, 11 and 12 h yielded recovery fractions that tended to be higher than the mean fractions after periods of either 4.5 ($p<0.05$) or 5 h.

5.4.5. Effects of equilibration periods on the recovery fraction of Nguni semen

Shorter equilibration periods (4.5, 5, 5.5, 6 and 6.5 h) applied to semen samples recorded mean recovery fractions of 0.41±0.17, 0.47±0.07, 0.51±0.15, 0.47±0.13 and 0.50±0.13. The recovery fractions resulting from equilibration periods of 4.5, 5, 5.5, 6 and 6.5 h were not significantly different from one another.

However, equilibrating semen samples for longer periods (7, 7.5, 8, 8.5, 9 10, 11 or 12 h) resulted in recovery fractions of 0.54±0.12, 0.54±0.13, 0.52±0.10, 0.53±0.12, 0.53±0.12, 0.49±0.18 and 0.51±0.10, respectively. The recovery fractions mentioned above were not significantly different from one another. The mean recovery fraction yielded by a 4.5 h total equilibration period was significantly lower ($p<0.05$) than recovery fractions yielded by longer equilibration periods of 7, 7.5, 8, 8.5, 9 and 10 h. Mean recovery fractions resulting from total equilibration periods of 5, 5.5 and 6.5 h were not significantly different from the mean recovery fractions yielded by total equilibration periods of 7, 7.5, 8, 8.5 or 9 10, 11 or 12 h. The mean recovery fraction resulting from 6 h of equilibration tended to be lower than the mean recovery fraction yielded by 8.5 h of total equilibration. However there were not significantly different from recovery fractions recorded for any of the other equilibration periods (7, 7.5, 8, 9 10, 11 or 12 h).

5.4.6. Effect of equilibration periods and breed on the differences in recovery fraction of bovine semen

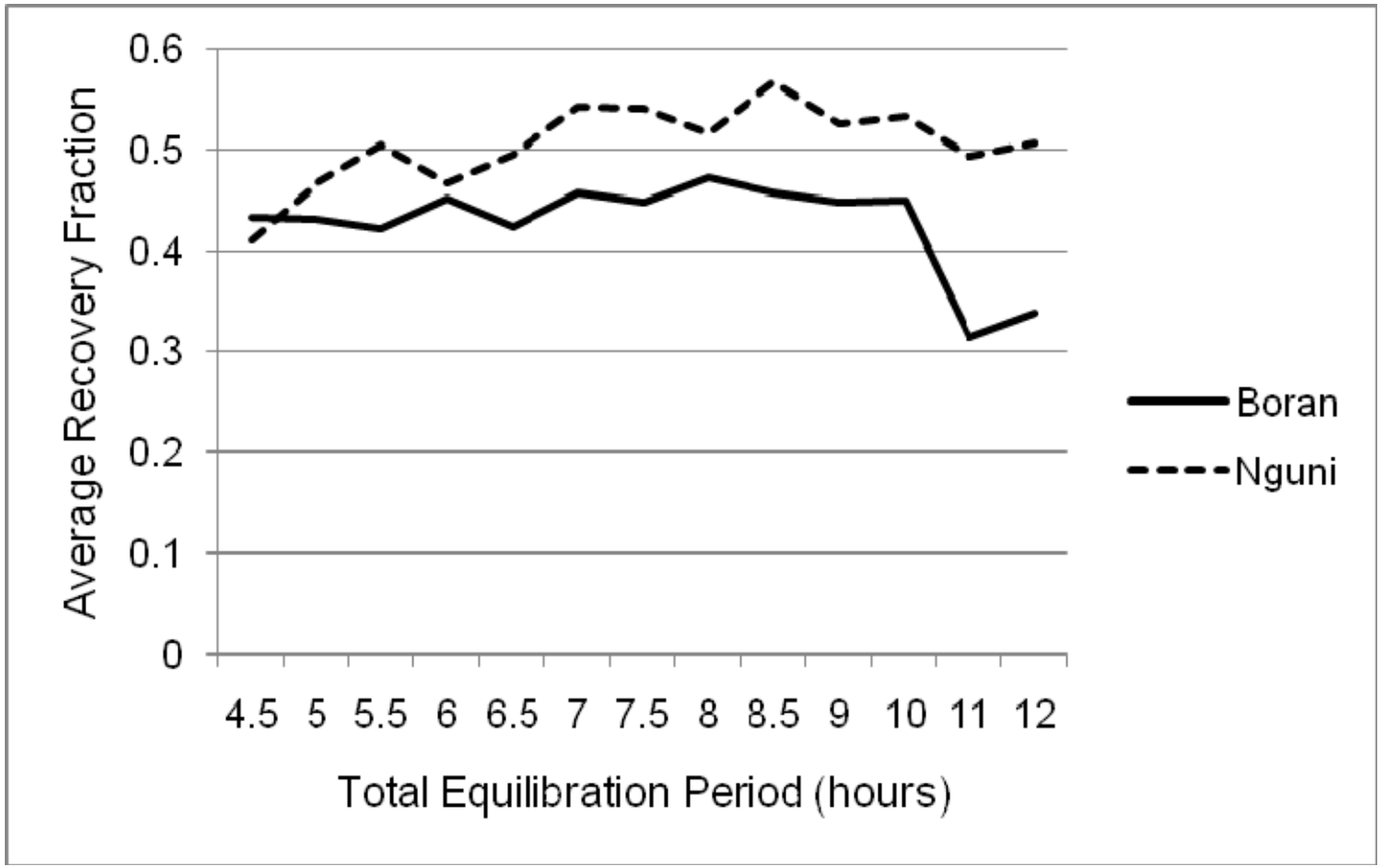
When comparing the resulting mean recovery fractions from the two cattle breeds after either 5 or 7 h total equilibration time, the Nguni breed had a significantly higher ($p<0.05$) mean recovery fraction. The Nguni also yielded mean recovery fractions that tended to be higher – compared to that of the Boran – after total equilibration periods of 7.5 and 8.5 h. After equilibration periods of 4.5, 5.5, 6, 6.5, 8, 9, 10, 11 or 12 h the difference between the mean recovery fractions recorded by the Boran and Nguni breeds were not significantly different.

TABLE 5.4: Effects of different equilibration periods on the mean (±SD) recovery fraction of Boran and Nguni semen

Total Equilibration Time (h)	BREED	
	Boran	**Nguni**
	Mean Recovery Fraction	Mean Recovery Fraction
4.5	$0.314^{aA} \pm 0.130$	$0.410^{a} \pm 0.166$
5	$_{a}0.338^{abA} \pm 0.123$	$_{b}0.468^{ab} \pm 0.065$
5.5	$0.434^{aBbc} \pm 0.046$	$0.506^{ab} \pm 0.154$
6	$0.431^{bBc} \pm 0.041$	$0.467^{abA} \pm 0.129$
6.5	$0.422^{aBbc} \pm 0.016$	$0.496^{ab} \pm 0.125$
7	$_{a}0.452^{c} \pm 0.037$	$_{b}0.544^{b} \pm 0.122$
7.5	$_{aA}0.424^{aBbc} \pm 0.053$	$_{aB}0.542^{b} \pm 0.125$
8	$0.457^{c} \pm 0.054$	$0.517^{b} \pm 0.102$
8.5	$_{aA}0.448^{bBc} \pm 0.064$	$_{aB}0.568^{bB} \pm 0.146$
9	$0.473^{c} \pm 0.048$	$0.526^{b} \pm 0.115$
10	$0.458^{c} \pm 0.058$	$0.533^{b} \pm 0.119$
11	$0.448^{bBc} \pm 0.118$	$0.494^{ab} \pm 0.184$
12	$0.450^{bBc} \pm 0.070$	$0.508^{ab} \pm 0.101$

^{a,b,c} = Means in the same column with different superscripts differ significantly ($p<0.05$)
a,b,c = Means in the same row with different superscripts differ significantly ($p<0.05$)
$^{A.B}$ = Means in the same column with different superscript letter tended to differ ($p<0.1$)
A.B = Means in the same row with different superscript letter tended to differ ($p<0.1$)

Figure 5.4: The relationship between the equilibration period and mean recovery fractions of cryopreserved semen from Boran and Nguni bulls



5.5. Effect of different cooling and glycerol equilibration periods on the post-thaw sperm motility of bovine semen

The results of the effect of the cooling period and glycerol equilibration period on the post-thaw sperm motility are summarized in Table 5.5. The general trend for the effect of different cooling periods on mean post-thaw motility of Boran and Nguni semen is further illustrated in Figure 5.5. The trend for the effect of different glycerol equilibration periods on the mean post-thaw sperm motility of Boran and Nguni semen is also illustrated in Figure 5.6.

5.5.1 Effect of different cooling periods on the post-thaw sperm motility of bovine semen

Overall semen samples subjected to longer cooling periods produced higher post-thaw sperm motility rates. The shortest ($p<0.05$) cooling period (4 h) yielded an overall mean post-thaw motility rate of 35.7±10.4% and the longest (differed from 4 h – $p<0.05$; but not significantly different from 5, 6 or 7 h) cooling period of 8 h yielded a mean post-thaw motility rate of 42.8±9.8%. Periods of 5, 6 and 7 h of cooling produced mean post-thaw motility rates of 40.7±9.7%, 41.2±8.5% and 41.5±10.5%, respectively. The mean post-thaw sperm motility resulting from 4 h of cooling was significantly lower ($p<0.05$) than the motilities for cooling for 5, 6, 7 or 8 h. Cooling periods of 5, 6, 7 and 8 h resulted in a post-thaw motility rates that were not significantly different from one another.

Smith and Merilan (1991) reported that a slow cooling rate (cooling semen to 5°C over 3 h, compared to a faster cooling rate where semen was cooled to 5°C within 0.5 h) was beneficial to the post-thaw survival of spermatozoa (34.5% vs 31.4%). Similarly Griffin (2004) also found a cooling period of 4 h to be superior to a 2 h cooling period.

Dhami and Sahni (1993) reported a 2 h cooling period to yield significantly higher ($p<0.01$) post-thaw motility rates than a 1 h cooling period. This is in agreement with the results from the present study which demonstrated a trend that a longer cooling period resulted in higher post-thaw sperm motility rates.

Subjecting semen samples to 30 minutes of glycerol equilibration after a cooling period of 4 h resulted in the lowest (significantly different; $p<0.05$) post-thaw motility rate (31.0±13.5%). A 6 h cooling period followed by the same equilibration period yielded a mean post-thaw sperm motility of 39.1±8.8%. Cooling periods of 5 h (40.0±10.7%), 7 h (41.3±10.8%) and 8 h (43.3±12.0%) produced increasing – although not significantly different – post-thaw motility rates. Significant differences ($p<0.05$) were recorded between the mean post-thaw motility of samples cooled for 4 and 5, 4 and 7, 4 and 8 h, as well as 6 and 7 h. There was a tendency towards a difference in the mean post-thaw motility rate of 4 and 6 h. Mean post-thaw motility rate from 5 and 6, 5 and 7, 5 and 8, 6 and 8 and also 7 and 8 h, were not significantly different.

With 60 minutes of glycerol equilibration, a cooling period of 4 h recorded the lowest (different from 8 h at $p<0.05$ and tended to differ from 6 and 7 h) mean post-thaw sperm motility rate (34.3±10.6%) and a period of 8 h the highest (no significant difference) motility (44.4±9.8%). Cooling periods of 5, 7 and 6 h produced the second lowest (39.9±10.1%), third highest (42.1±10.4%) and second highest (all not significant) mean post-thaw sperm motility (42.3±9.6%) respectively.

There was a significant difference ($p<0.05$) between the post-thaw motility resulting from cooling periods of 4 and 8 h, and a tendency towards significance between the mean post-thaw motility of 4 and 6 h, as well as 4 and 7 h. The mean post-thaw motility resulting from 5, 6, 7 and 8 h cooling periods was not significantly different from one another. The post-thaw motility following 4 and 5 h of cooling also recorded no significant differences, when compared.

Cooling semen for periods of 4 and 8 h respectively resulted in the lowest (no significantly different) (36.6±8.3%) and highest (42.8±10.1%) post-thaw motility rates, when combined with a 120 minute glycerol equilibration period. Periods of 5 h (41.4±10.6%), 7 h (42.4±8.7%) and 6 h (42.5±8.3%) produced increasing (no significant difference) mean post-thaw motility rates.

Using a 240 minute glycerol equilibration period with cooling periods of 7 and 5 h resulted in the lowest (not significantly different) (40.0±13.3%) and highest (no significant difference) (41.4±8.6%) mean post-thaw motility. Both 4 and 6 h of cooling

recorded the second highest (41.0±7.2% and 41.0±8.2%) post-thaw motility rate, while a period of 8 h yielded the second lowest (40.7±8.1%) mean post-thaw motility rate (not significantly different).

There were also no significant differences between the post-thaw motility resulting from the respective cooling periods, when either a 120 or 240 minute glycerol equilibration period was employed.

5.5.2. Effects of different glycerol equilibration periods on the post-thaw sperm motility of bovine semen

Over all the cooling periods, subjecting semen samples to a glycerol equilibration period of 30 minutes once again produced the lowest post-thaw motility (38.9±11.6%) not significant. Equilibration periods of 60 (40.6±10.3%), 240 (40.8±9.0%) and 120 (41.1±9.2%) minutes resulted in increasing post-thaw motility. There was no significant difference between any of the mean post-thaw motility rates yielded by the different equilibration periods.

Gilbert and Almquist (1978) reported on the effects of glycerol equilibration periods of 0, 3 or 9 h, and found that a 0 h period resulted in a significantly lower mean post-thaw motility (55.3 ±2.4%) compared to either a 3 h (57.7±2.2%) or 9 h period (58.3% ±1.6%). The resulting sperm motility after 3 or 9 h of glycerol equilibration did not differ significantly.

O'Dell and Hurst (1955) stated that a 0.5 h glycerol equilibration period recorded a significantly higher ($p<0.05$) post-thaw motility rate, compared to an 18 h glycerol equilibration period. In contrast, Martin and Emmens (1961) found the post-thaw motility after a 30 minute glycerol equilibration period (54.2%) to be significantly lower ($p<0.001$) than after an 18 h glycerol equilibration period (66.0%). In the present study there was no significance in the differences between the mean post-thaw motility yielded after glycerol equilibration periods of either 30 minutes of longer periods of 60, 120 or 240 minutes. Hill *et al.* (1959) found a 0.5 h glycerol equilibration period yielded higher post-thaw motility than an 18 h glycerol equilibration period for ram semen.

Dhami and Sahni (1993) also found that a 2 h glycerol equilibration period yielded a significantly higher ($p<0.01$) mean post-thaw motility, than a 1 h glycerol equilibration period. Although the post-thaw motility yielded by a 2 h cooling period in the present study, was slightly higher than that yielded by a 1 h cooling period, the difference was not significant.

Berndtson and Foote (1969) reported that a 10 second glycerol equilibration period resulted in a significantly higher post-thaw motility (43%) compared to either a 30 minute (38%) or 6 h (37%) glycerol equilibration period. It was suggested that the harmful effects of glycerol had not occurred after 10 seconds of equilibration, but sufficient penetration had taken place to protect the spermatozoa. In another study by Berndtson and Foote (1972) it was found that a 10 second glycerol equilibration period to be superior to periods of 10, 20, 30 minutes or 6 h. Almlid and Johnson (1988) studied the effects of glycerol equilibration periods on boar semen and established that there were no significant differences in the motility of spermatozoa equilibrated for 0.5, 2, 5, 15 or 75 minutes. Leite *et al.* (2010) reported that glycerol equilibration periods of either 2 or 4 h resulted in significantly higher motility rates than a no glycerol equilibration period. A 4 h glycerol equilibration period yielded superior post-thaw sperm motility than glycerol equilibration periods of either 2 of 6 h (Tuli *et al.*, 1981). It was also found that a glycerol equilibration period of either 4 or 6 h to be optimum for the freezing bovine semen (Griffin, 2004).

Wiggin and Almquist (1974) stated that a 2 h glycerol equilibration period was optimum for semen frozen in straws, and that glycerol equilibration periods exceeding 4 h were not recommended. Saroff and Mixner (1955) studied glycerol equilibration periods of 2, 6, 12 and 18 h and concluded that there was a direct relationship between post-thaw sperm motility and the glycerol equilibration period. Sullivan and Mixner (1963) also researched glycerol equilibration periods of 2, 6, 12 and 18 h and found that a 2 h period was inferior to any of the longer glycerol equilibration periods (6, 12 or 18 h).

When the effects of glycerol equilibration periods of 2, 3, 4, 5, 6, 7, 8 and 9 h on the post-thaw motility of African buffalo semen were studied, no significant differences between the different glycerol equilibration periods were recorded (Herold e*t al.*, 2006).

When allowing semen to cool for a period of 4 h prior to glycerol addition, the lowest (differed ($p<0.05$) for 240 minutes) the mean post-thaw motility of 31.0±13.5% was the result of a 30 minute glycerol equilibration period. Increasing post-thaw motility resulted from equilibration periods of 60 (34.3±10.6%), 120 (36.6±8.3%) and 240 minutes (41.0±7.2%) respectively. The only significant difference ($p<0.05$) observed within a 4 h cooling period was with a glycerol equilibration period of 30 minutes, that resulted in a significantly lower post-thaw motility than that of 240 minutes.

With 5 h of cooling there were no significant differences between the post-thaw motility rates resulting from glycerol equilibration periods of 30, 60, 120 and 240 minutes. The mean post-thaw sperm motility recovered after 5 h of cooling and 60 minutes of equilibration was the lowest (not significant) (39.9±10.1%) and the mean post-thaw motility rate after 30 minutes, the second lowest (not significant) (40.0±10.7%). Cooling for 120 and 240 minutes both resulted in the highest (although not significant) mean post-thaw motility rate (41.4±10.6% and 41.4±8.6%).

Following a 6 h cooling period, glycerol equilibration times of 30 and 240 minutes resulted in the lowest (39.1±8.8%) and second lowest mean post-thaw motility rates (41.0±8.2%) – not significant. Equilibration periods of 120 and 60 minutes yielded the highest (42.5±8.3%) and second highest (both not significantly different) mean post-thaw motility rates (42.3±9.6%), respectively. There was no significant difference in the differences between any of the mean post-thaw motility rates yielded by the different equilibration periods.

When combined with a 7 h cooling period, equilibration periods of 240 (40.0±13.3%), 30 (41.3±10.8%), 60 (42.1±10.4%) and 120 minutes (42.4±8.7%) produced increasing (no significant difference) post-thaw motility. There were no significant differences recorded between the mean post-thaw motility rates of the different equilibration periods.

After a cooling period of 8 h, the lowest not significant difference (40.7±8.1%) and highest (44.4±9.8%) mean post-thaw motility were the result of 240 and 60 minutes of glycerol equilibration, respectively. Equilibration for 30 and 120 minutes produced the second highest (43.3±12.0%) and second lowest (both not significant differences) (42.8±10.1%) mean post-thaw motility rates. The differences between

the mean post-thaw motility rates yielded by the different periods of equilibration were not significantly significant.

TABLE 5.5: The overall effect of different cooling and glycerol equilibration periods on the mean (±SD) post-thaw sperm motility rate of bovine semen

GLYCEROL EQUILIBRATION PERIOD (minutes)	COOLING PERIOD (h) 4	5	6	7	8	Overall mean (±SD)
30	31.0^{aA} ±13.5	40.0^{eh} ±10.7	39.1^{aBeg} ±8.8	41.3^{h} ±10.8	43.3^{ehi} ±12.0	**38.9^{a} ±11.6**
60	34.3^{abA} ±10.6	39.9^{bef} ±10.1	42.3^{bBfg} ±9.6	42.1^{bBfh} ±10.4	44.4^{fi} ±9.8	**40.6^{a} ±10.3**
120	36.6^{abc} ±8.3	41.4^{ce} ±10.6	42.5^{cg} ±8.3	42.4^{ch} ±8.7	42.8^{ci} ±10.1	**41.1^{a} ±9.2**
240	41.0^{bd} ±7.2	41.4^{de} ±8.6	41.0^{dg} ±8.2	40.0^{dh} ±13.3	40.7^{di} ±8.1	**40.8^{a} ±9.0**
Overall mean (±SD)	**35.7^{a} ±10.4**	**40.7^{b} ±9.7**	**41.2^{b} ±8.5**	**41.5^{b} ±10.5**	**42.8^{b} ±9.8**	

a,b,c Means in the same row or column with different superscripts differ significantly (p<0.05)
a,b,c Means in the same row or column with different superscripts differ significantly (p<0.05)
A.B Means in the same row or column with different superscript letter tended to differ (p<0.1)
A.B Means in the same row or column with different superscript letter tended to differ (p<0.1)

Figure 5.5: The relationship between the cooling period and mean post-thaw sperm motility of cryopreserved bovine semen

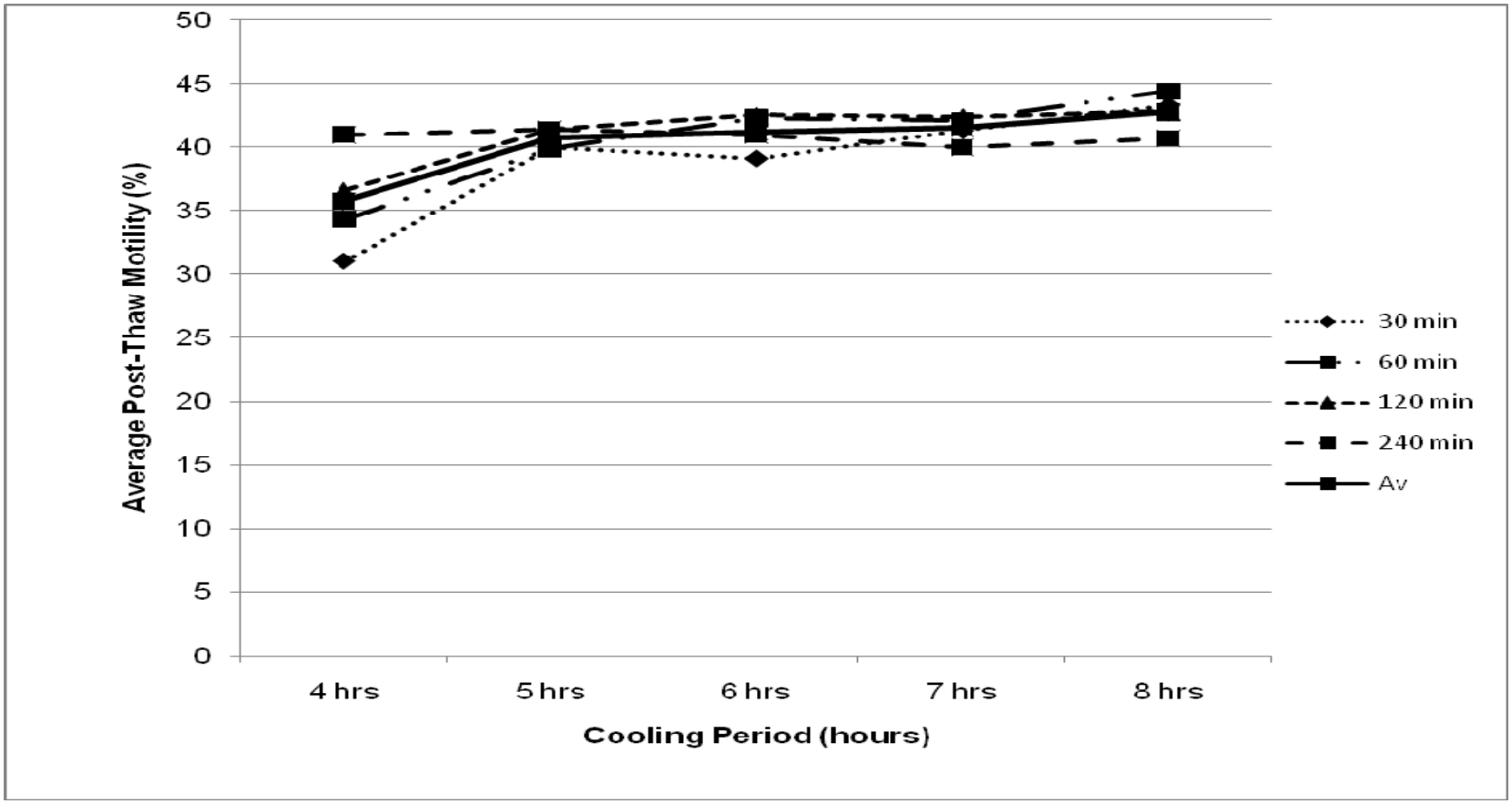
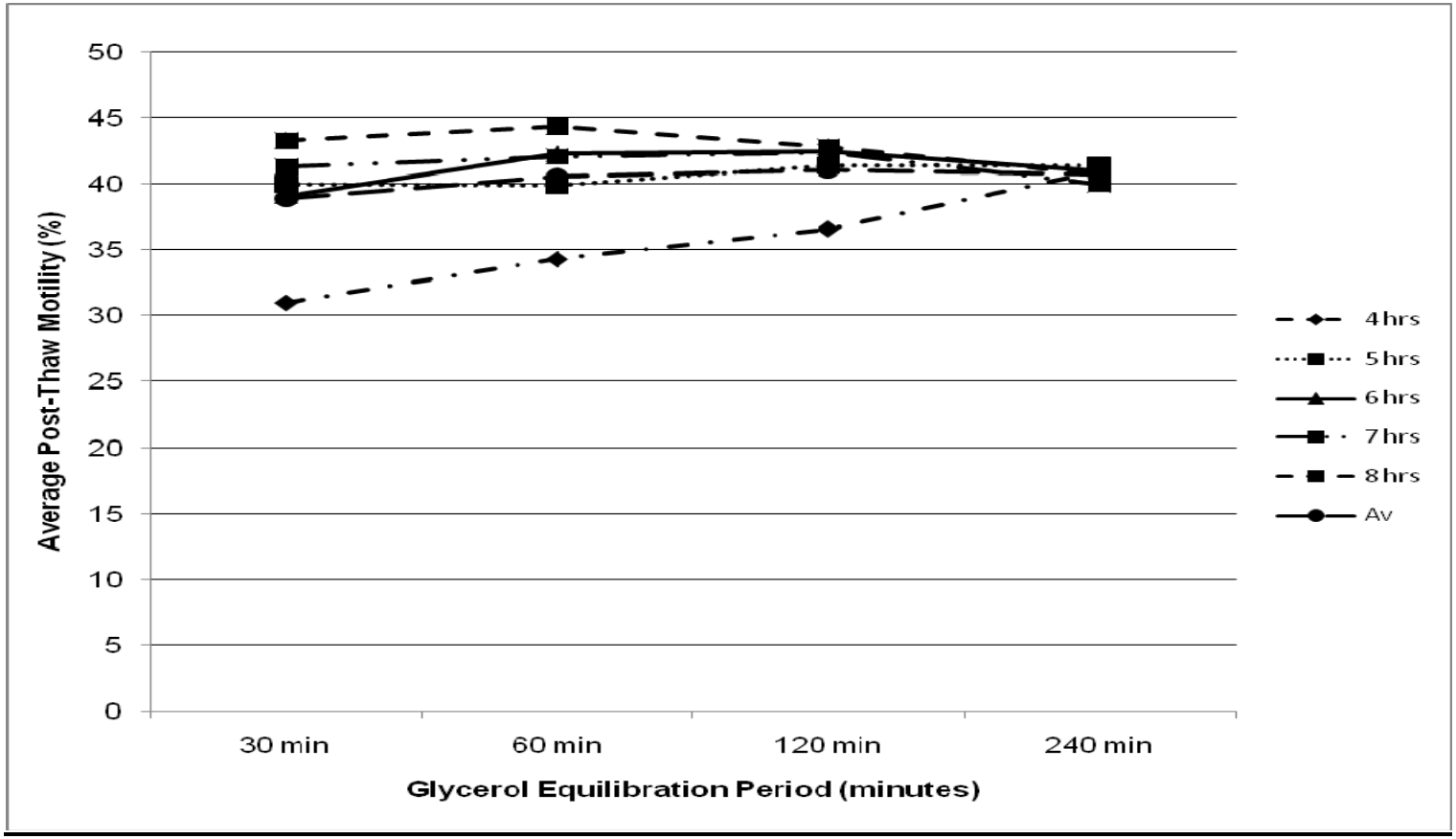


Figure 5.6: The relationship between the glycerol equilibration period and mean post-thaw sperm motility of cryopreserved bovine semen



5.6. Effect of different cooling and glycerol equilibration periods on the recovery fractions of bovine semen

The results of the effect of the different cooling and glycerol equilibration periods on the recovery fractions are summarized in Table 5.6. The general trend for the effect of different cooling periods on mean recovery fraction of bovine semen is illustrated in Figure 5.7, while the effect of different glycerol equilibration periods on the mean recovery fraction of bovine semen is illustrated in Figure 5.8.

The author could not find any previous studies using mean recovery fraction of bovine semen as a variable.

5.6.1 Effect of different cooling periods on the recovery fraction of bovine semen

Overall, the longer cooling periods produced a higher recovery fraction. The lowest fraction, 0.42±0.12 (4 h of cooling) differed significantly ($p<0.05$) from the recovery fractions resulting from the longer cooling periods. Cooling periods of 5, 6, 7 and 8 h recorded mean recovery fractions of 0.48±0.10, 0.48±0.09, 0.49±0.11 and 0.50±0.10. These mean recovery fractions again were not significantly different from one another.

Subjecting semen samples to a 30 minute glycerol equilibration period after the respective cooling periods, recorded a mean recovery fraction (0.36±0.15) for a 4 h cooling period, to be significantly smaller ($p<0.05$) than that of the increasing recovery fractions produced by a 5 h (0.47±0.11), 6 h (0.46±0.09), 7 h (0.48±0.11) or 8 h (0.51±0.12) cooling period. The recovery fractions resulting from cooling periods of 5, 6, 7 and 8 h were not significantly different from one another.

With 60 minutes of glycerol equilibration, increasing recovery fractions were produced by cooling periods of 4, 5, 7, 6 and 8 h. There were significant differences ($p<0.05$) between the mean recovery fractions resulting from cooling times of 4 h (0.40±0.12) and 6 h (0.50±0.10), as well as 4 and 8 h (0.52±0.10). There was also a tendency towards a significant difference when comparing the recovery fractions of the 4 and 7 h (0.49±0.11) cooling periods. The mean recovery fraction (0.47±0.10) yielded by a 5 h cooling period was not significantly different from any other cooling

period used. Mean recovery fractions resulting from cooling periods of 6, 7 and 8 h were not significantly different from each other.

When combining a 120 minute equilibration period with the respective cooling periods, resulted in mean recovery fractions of 0.43±0.09, 0.49±0.11, 0.50±0.08, 0.50±0.09 and 0.50±0.11, when semen was cooled for 4, 5, 6, 7 or 8 h, respectively. There were no significant differences between any of the resulting recovery fractions.

Cooling periods of 5 and 7 h resulted in the highest (no significant differences) (0.49±0.09) and lowest (0.47±0.15) recovery fractions, when combined with a 240 minute glycerol equilibration. Combining a glycerol equilibration period with cooling periods of 4, 6 and 8 h respectively, resulted in mean recovery fractions of 0.48±0.08, 0.48±0.09 and 0.48±0.09. There were no significant differences between the respective mean recovery fractions when a 240 minute glycerol equilibration period was employed.

5.6.2 Effects of different glycerol equilibration periods on the recovery fraction of bovine semen

Overall, a glycerol equilibration period of 30 (0.46±0.13) minutes yielded the lowest mean fraction while periods of 60 (0.48±0.11), 120 (0.48±0.10) and 240 minutes (0.48±0.10) all resulted in the highest recovery fraction (not significant). The overall recovery fractions were not significantly different.

When a cooling period of 4 h was combined with several glycerol equilibration periods, a significant difference ($p<0.05$) between the recovery fractions of semen cooled for 30 and 240 minutes was recorded. The recovery fractions of 60 and 240 minutes were not significantly different. When cooling periods of 30 and 60, 30 and 120, 60 and 120 and also 120 and 240 minutes were compared, the resulting recovery fractions were not significantly different. As the equilibration period lengthened, the mean recovery fraction also increased. Periods of 30, 60, 120 and 240 minutes produced mean recovery fractions of 0.36±0.15, 0.40±0.12, 0.43±0.09 and 0.48±0.08.

With 5 h of cooling there were no significant differences between the mean recovery fractions, resulting from the respective equilibration times. The recovery fractions recovered after 30 and 60 minutes of equilibration and 5 h of cooling were the lowest (not significantly different) (0.47±0.11 and 0.47±0.10) and the mean recovery fractions after 120 and 240 minutes, the highest (not significantly different) (0.49±0.11 and 0.49±0.09).

When employing a 6 h cooling period, there were no significant differences between the recovery fractions resulting from the respective glycerol equilibration times. Equilibration periods of 60 and 120 minutes yielded the highest (not significantly different) (0.50±0.10 and 0.50±0.08) mean recovery fractions. Equilibration for 30 and 240 minutes again resulted in the lowest (0.46±0.09) and second lowest (not significantly different) mean recovery fractions (0.48±0.09).

With 7 h of cooling, 240 minutes of glycerol equilibration resulted in the lowest mean recovery fraction (0.47±0.15) while equilibration for 120 minutes produced the highest mean recovery fraction (0.50±0.09) (not significant). Equilibration periods of 30 and 60 minutes yielded the second lowest (not significantly different) (0.48±0.11) and second highest (not significantly different) (0.49±0.109) mean recovery fractions. Differences between the recovery fractions from the respective equilibration periods were not significantly different

When combined with a cooling period of 8 h, there were no significantly differences between mean recovery fractions resulting from the different glycerol equilibration periods. Equilibrating semen for 240 minutes resulted in the lowest mean recovery fraction (0.48±0.09) while a 60 minute equilibration period produced the highest recovery fraction (0.52±0.10) (both not significant). Periods of 120 and 30 minutes yielded the second lowest (0.50±0.11) and second highest (not significantly different) (0.51±0.12) mean recovery fractions.

TABLE 5.6: The overall effect of different cooling and glycerol equilibration periods used in Experiment 2 on the mean (±SD) recovery fraction of bovine semen

GLYCEROL EQUILIBRATION PERIOD (min)	COOLING PERIOD (h) 4	5	6	7	8	Overall mean (±SD)
30	0.36^{a} ±0.15	0.47^{e} ±0.11	0.46^{eg} ±0.09	0.48^{eh} ±0.11	0.51^{ei} ±0.12	**$0.46^{\underline{a}}$ ±0.13**
60	0.40^{abA} ±0.12	0.47^{bef} ±0.10	0.50^{fg} ±0.10	0.49^{bBfh}±0.11	0.52^{fi} ±0.10	**$0.48^{\underline{a}}$ ±0.11**
120	0.43^{abc} ±0.09	0.49^{ce} ±0.11	0.50^{cg} ±0.08	0.50^{ch} ±0.09	0.50^{ci} ±0.11	**$0.48^{\underline{a}}$ ±0.10**
240	0.48^{bBd} ±0.08	0.49^{de} ±0.09	0.48^{dg} ±0.09	0.47^{dh} ±0.15	0.48^{di} ±0.09	**$0.48^{\underline{a}}$ ±0.10**
Overall mean (±SD)	**$0.42^{\underline{a}}$ ±0.12**	**$0.48^{\underline{b}}$ ±0.10**	**$0.48^{\underline{b}}$ ±0.09**	**$0.49^{\underline{b}}$ ±0.11**	**$0.50^{\underline{b}}$ ±0.10**	

^{a,b,c} Means in the same row or column with different superscripts differ ($p<0.05$)
$^{\underline{a.b.c}}$ Means in the same row or column with different superscripts differ ($p<0.05$)
$^{A.B}$ Means in the same row or column with different superscripts tended to differ ($p<0.1$)
$^{\underline{A.B}}$ Means in the same row or column with different superscripts tended to differ ($p<0.1$)

Figure 5.7: The relationship between the cooling period and mean recovery fraction of cryopreserved bovine semen

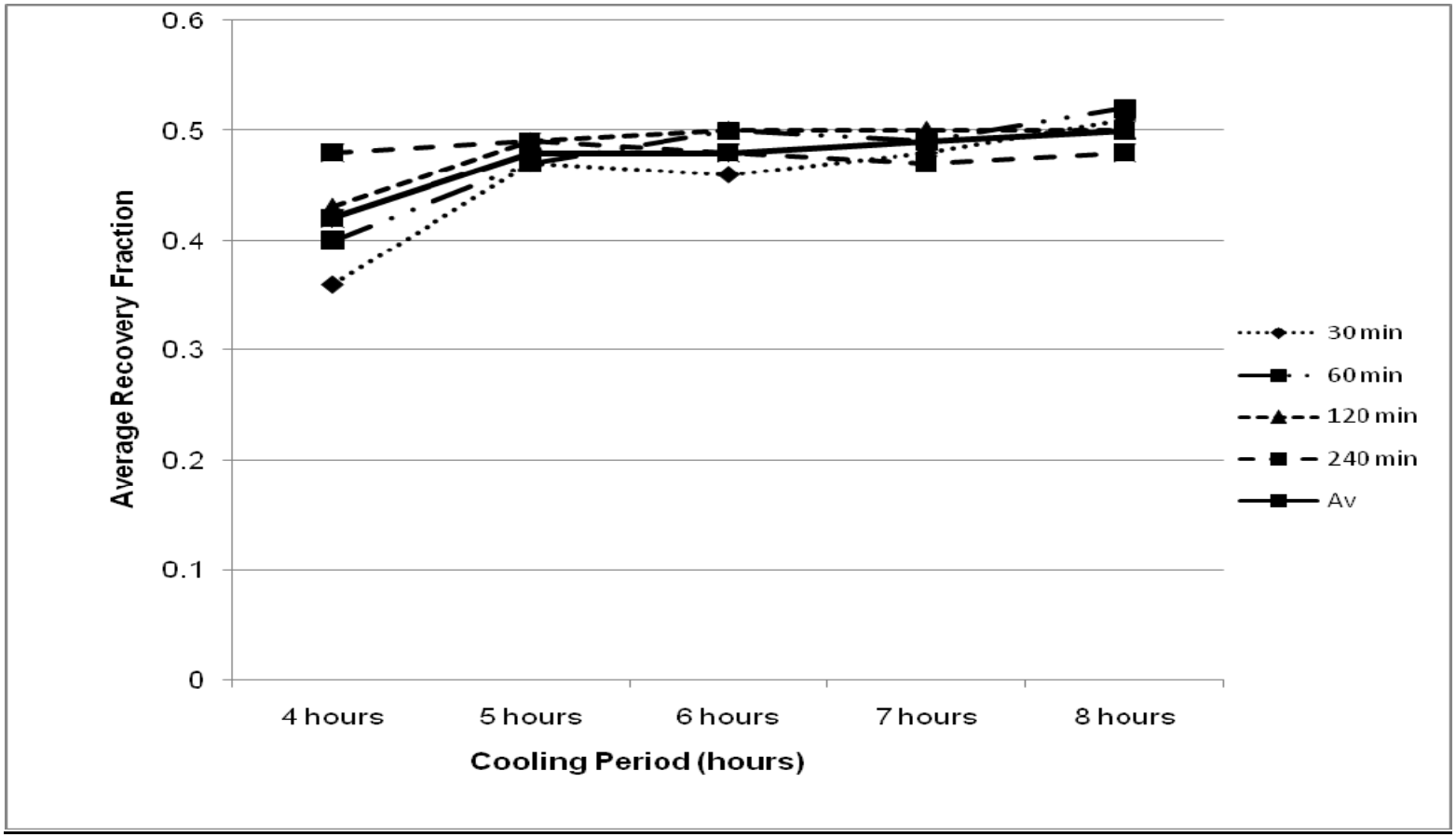
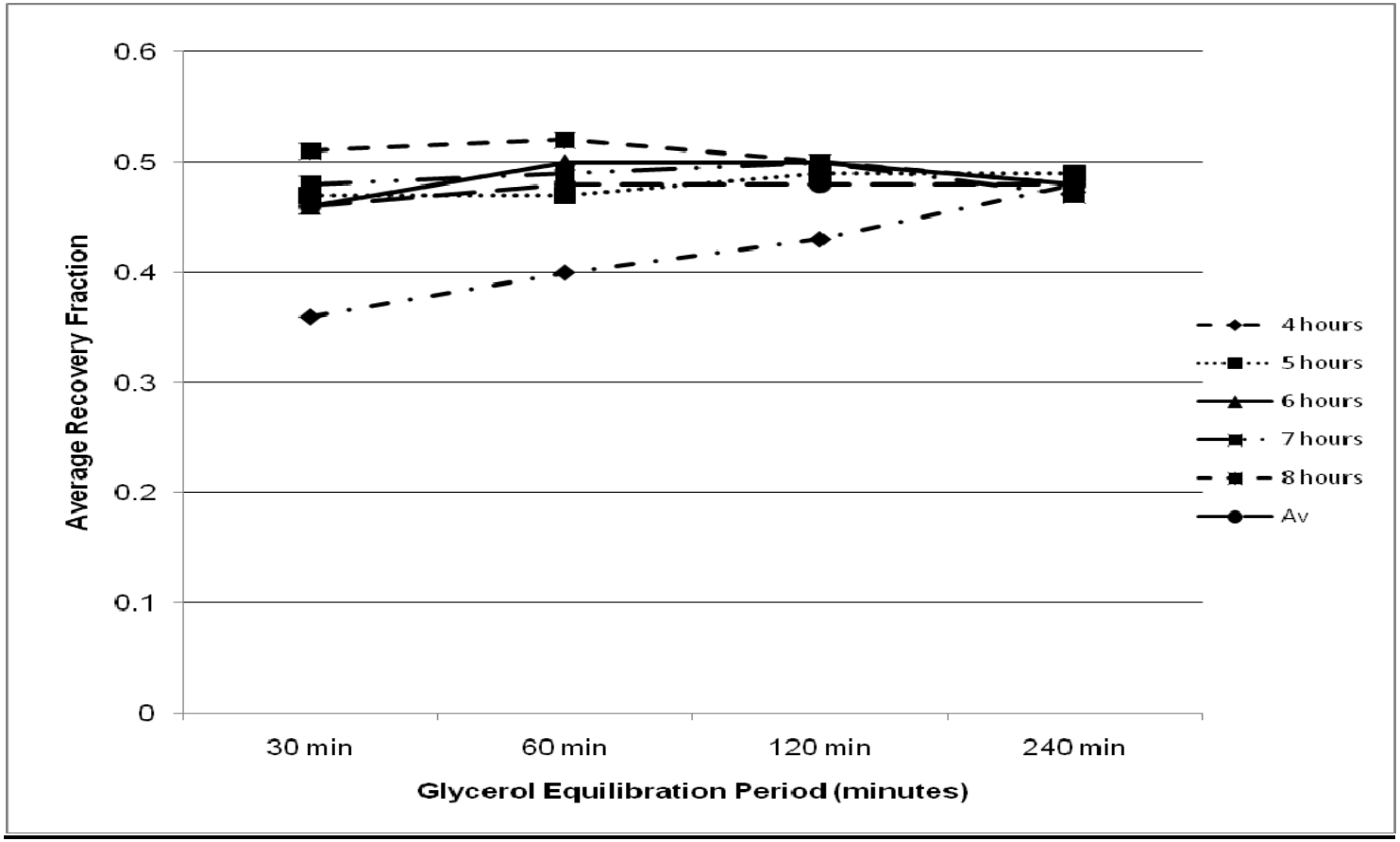


Figure 5.8: The relationship between the glycerol equilibration period and mean recovery fraction of cryopreserved bovine semen



5.7 Effect of different cooling periods on the post-thaw sperm motility rates of Boran and Nguni semen

The results of the effect of different cooling periods on the post-thaw sperm motility rates of Boran and Nguni semen are summarized in Table 5.7. The general trend for the effect of different cooling periods on mean post-thaw sperm motility rate of Boran semen is also illustrated in Figure 5.9, together with the trend of Nguni, semen is illustrated in Figure 5.10.

The author could not find any previous studies comparing frozen-thawed semen from Boran and Nguni bulls.

5.7.1 Effects of different cooling periods on the post-thaw sperm motility rate of Boran semen

When cooling periods of 4, 5, 6, 7 and 8 h were compared, over all equilibration periods, the Boran recorded a mean post-thaw sperm motility rates of 31.6±8.7%, 36.7±2.7%, 37.4±2.3%, 37.4±6.0% and 38.2±4.6% for the respective periods. The post-thaw motility resulting from a 4 h cooling period tended to differ from the post-thaw motility after 5, 6 and 7 h. There was also a significant difference ($p<0.05$) between the post-thaw motility recorded by cooling periods of 4 or 8 h. Cooling periods of 5, 6, 7 and 8 h resulted in a mean post-thaw motility that was not significantly different from one another.

So for example, Dhami and Sahni (1993) found a 2 h cooling period to yield significantly higher ($p<0.01$) post-thaw motility rates than a 1 h cooling period. Smith and Merilan (1991) determined that a slow cooling rate (cooling semen to 5°C over 3 h, compared to a faster cooling rate where semen was cooled to 5°C within 0.5 h) was beneficial for the post-thaw survival of spermatozoa. Griffin (2004) also found a cooling period of 4 h to be superior to a 2 h cooling period.

With a glycerol equilibration period of 30 minutes, the Boran's lowest (not significantly different from 5, 6 and 7 h, tended to differ from 8 h). Mean post-thaw motility (26.0±10.8) was the result of a 4 h cooling period. An increase in the mean post-thaw motility resulted from equilibration periods of 6 h (35.0±0.0%), 7 h

(35.2±4.0%), 5 h (35.8±3.1%) and 8 h (37.0±5.15), respectively. The post-thaw motilities after glycerol equilibration periods of 4 and 8 h tended to be different. Comparing mean rates from periods of 5, 6, 7 and 8 h showed no significant difference between these post-thaw motility rates. There was also no significant differences between the mean post-thaw motility rates for periods of 4 and 5 h, 4 and 6 h, as well as 4 and 7 h.

With a cooling period of 4 h and 60 minutes of glycerol equilibration, the Boran post-thaw motility (28.0±10.4%) that tended to be different, compared to the cooling periods of 6 h (38.6±2.2%), 7 h (38.0±5.7%) and 8 h (39.8±3.0%). Although a 5 h cooling period resulted in a lower post-thaw sperm motility (36.2±1.6%) when compared to 6, 7 and 8 h, this difference was not significant. The mean post-thaw motility rates resulting from 6, 7 or 8 h of cooling were also not significant.

When variable cooling periods preceded a 120 minute glycerol equilibration period, increasing the post-thaw motility resulted from 4 h (35.2±4.0%), 5 h (36.2±1.6%), 6 h (38.6±2.2%), 8 h (38.6±5.5%) and 7 h (39.2±4.3%), respectively. There was only a tendency towards significant difference between the post-thaw motility after 4 and 7 h of cooling. Similarly there were no significant differences between the respective post-thaw motility rates when cooling periods of 5, 6 or 8 h were compared to a 4 h period. Cooling periods of 5, 6, 7 and 8 h resulted in a mean post-thaw motility rates that were not significantly different from one another.

When combined with a glycerol equilibration period of 240 minutes, 4 (37.0±3.8%) or 7 h (37.0±9.8%) of cooling yielded the lowest (no significant differences) post-thaw motility rates. Cooling semen for 6 h (37.2±2.2%) or 8 h (37.2±5.2%) resulted in the second lowest post-thaw motility rates, while a 5 h cooling period yielded the highest post-thaw motility rates (38.4±3.8%) (both not significant). With an equilibration period of 240 minutes, the Boran recorded no significant differences between the mean post-thaw motility rates for the different cooling periods.

5.7.2 Effects of different cooling periods on the post-thaw sperm motility of Nguni semen

Over all the equilibration periods, a 4 h cooling period yielded an mean post-thaw motility rate that was significantly lower ($p<0.05$) than the post-thaw motility after 8 h. Mean post-thaw motility rates after cooling periods of 6 and 7 h, tended to differ from the mean post-thaw motility after 4 h of cooling. There were no significant differences recorded between the mean post-thaw motility rates after cooling periods of 5, 6, 7 and 8 h, as well as periods of 4 and 5 h. An increasing mean post-thaw sperm motility of 39.9±10.5%, 44.7±12.3%, 45.1±10.6%, 45.6±12.5% and 47.5±11.5% were the result following 4, 5, 6, 7 and 8 h of cooling, respectively.

Smith and Merilan (1991) reported that a slow cooling rate (cooling semen to 5°C over 3 h, compared to a faster cooling rate where semen was cooled to 5°C within 0.5 h) was beneficial for the post-thaw survival of spermatozoa. It was also found that a 2 h cooling period yielded a significantly higher ($p<0.01$) mean post-thaw motility, compared to a 1 h cooling period (Dhami and Sahni, 1993). Griffin (2004) found a cooling period of 4 h to be superior to a 2 h cooling period.

With a glycerol equilibration period of 30 minutes, there was a significant difference ($p<0.05$) between the highest (49.6±14.1%) and lowest (36.0±15.2%) mean post thaw motility rates resulting from cooling periods of 8 and 4 h, respectively. There was a tendency towards significant differences regarding the mean post-thaw motility rates after 4 and 7 (47.4±12.5%) h of cooling. Cooling periods of 5, 6, 7 and 8 h resulted in post-thaw motility rates that were not significantly different from one another. A cooling period of 4 h resulted in post-thaw motility that was not significantly different from that of 5 or 6 h of cooling. Cooling semen for 6 h (43.2±11.6%) or 5 h (44.2±14.3%) produced the second and third lowest (no significant differences) mean post-thaw motility rates, respectively.

With a 60 minute glycerol equilibration period, longer cooling periods resulted in increasing the mean post-thaw motility. Cooling semen for 4, 5, 6, 7 and 8 h prior to glycerol equilibration recorded mean post-thaw motility rates of 40.6±6.7%, 43.6±13.9%, 46.0±12.9%, 46.2±13.0% and 49.0±12.4%. The differences between

the mean post-thaw motility rates yielded by the different periods of equilibration were not significantly significant.

Cooling periods of 4 h (38.0±11.5%), 7 h (45.6±11.2%), 6 h (46.4±10.6%), 5 h (46.6±13.5%) and 8 h (47.0±12.6%), followed by 120 minutes of glycerol equilibration produced, increasing the mean post-thaw motility rates. There were no significant differences between any of the mean post-thaw motility rates.

There were no significant differences between the resulting mean post-thaw motility rates of the Nguni when an equilibration period of 240 minutes was implemented. Cooling for periods of 7 h (43.0±16.8%), 8 h (44.2±9.5%), 5 h (44.4±11.4%), 6 h (44.8±10.6%) and 4 h (45.0±7.9%) resulted in increasing mean post-thaw motility rates.

5.7.3 Effect of different cooling periods and breed on the post-thaw motility of bovine semen

Comparing the mean post-thaw motility rates of the two breeds over all the equilibration periods, significant differences ($p<0.05$) were recorded for all four cooling periods. The cooling periods of 4, 5, 6, 7 and 8 h resulted in mean post-thaw motility rates of 31.6±8.7%, 36.7±2.7%, 37.4±2.3%, 37.4 ±6.0% and 38.2±4.6% for the Boran as well as 39.9±10.5%, 44.7±12.3%, 45.1±10.6%, 45.6±12.5% and 47.5±11.5% for the Nguni breed.

When a glycerol equilibration period of 30 minutes was followed the various cooling periods, there were no significant differences between the mean post-thaw motility rates, resulting from the two breeds after cooling periods of 5, 6, 7 and 8 h. There was however tendency towards significant difference between the mean post-thaw motility rates of the two breeds after 4 h of cooling. After a cooling period of 4 h the mean post-thaw sperm motility rates recorded by the Nguni and Boran bulls were 36.0±15.2% and 26.0±10.8%, respectively. The Boran thus produced mean post-thaw motility rates of 35.8±3.1%, 35.0±0.0%, 35.2±4.0% and 37.0±5.1% after 5, 6, 7 and 8 h of equilibration, while the Nguni recorded mean post-thaw motility rates of

44.2±14.3%, 43.2±11.6%, 47.4±12.5% and 49.6±14.1% after the same equilibration periods.

There were no significant differences between the mean post-thaw motility rates when a 60 minute glycerol equilibration period was applied in combination with cooling periods of 5, 6, 7 or 8 h. After a 4 h cooling period there was a significant difference ($p<0.05$) between the post-thaw motility rates for the two breeds. The mean post-thaw motility rates from the Nguni breed were once again higher (not significantly different after 5, 6, 7 and 8 h; differed ($p<0.05$) after 4 h), than the Boran mean post-thaw motility rates after the corresponding equilibration periods. For the Nguni periods of 4 h (40.6±6.7%), 5 h (43.6±13.9%), 6 h (46.0±12.9%), 7 h (46.2±13.0%) and 8 h (49.0±12.4%) resulted in increasing mean post-thaw motility rates. For the Boran equilibration periods of 4 (28.0±10.4%), 5 (36.2±1.6%), 7 (38.0±5.7%), 6 (38.6±2.2%) and 8 (39.8±3.0%) also resulted in increasing mean post-thaw motility rates.

With a glycerol equilibration period of 120 minutes, following 4, 6, 7 or 8 h cooling periods, no significant differences between the mean post-thaw motility rates of the two breeds were recorded. Means for the Boran and Nguni did however tended to differ after a cooling period of 5 h. Cooling semen for 4, 5, 6, 7 and 8 h respectively resulted in mean post-thaw motility rates of 35.2±4.0%, 36.2±1.6%, 38.6±2.2%, 39.2±4.3% and 38.6±5.5% for the Boran, as well as 38.0±11.5%, 46.6±13.5%, 46.4±10.6%, 45.6±11.2% and 47.0±12.6% for the Nguni.

Combining a 240 minute glycerol equilibration period with the different cooling periods recorded no significant differences between the mean post-thaw motility rates from the two breeds. For the Boran, cooling for periods of 4 h (37.0±3.8%) and 7 h (37.0±9.8%) resulted in the lowest post-thaw motility, while 6 h (37.2±2.2%) and 8 h (37.2±5.2%) of cooling recorded the second lowest post-thaw motility rates and 5 h (38.4±3.8%) yielded the highest mean post-thaw motility rate (all not significant). For the Nguni, periods of 7 h (43.0±16.8%), 8 h (44.2±9.5%), 5 h (44.4±11.4%), 6 h (44.8±10.6%) and 4 h (45.0±7.9%) recorded increasing mean post-thaw motility rates, although not significant.

TABLE 5.7: The between and within breed effect of different cooling periods, compared within several glycerol equilibration periods on the mean (±SD) post-thaw sperm motility of bovine semen

GLYCEROL EQUILIBRATION PERIOD	BREED	COOLING PERIOD 4	5	6	7	8
30	B	$_{aA}26.0^{aA}$ ±10.8	35.8^{a} ±3.1	35.0^{a} ±0.0	35.2^{a} ±4.0	37.0^{aB} ±5.1
	N	$_{aB}36.0^{aA}$ ±15.2	44.2^{ab} ±14.3	43.2^{ab} ±11.6	47.4^{aBb} ±12.5	49.6^{b} ±14.1
60	B	$_{a}28.0^{aA}$ ±10.4	36.2^{a} ±1.6	38.6^{aB} ±2.2	38.0^{aB} ±5.7	39.8^{aB} ±3.0
	N	$_{b}40.6^{a}$ ±6.7	43.6^{a} ±13.9	46.0^{a} ±12.9	46.2^{a} ±13.0	49.0^{a} ±12.4
120	B	35.2^{aA} ±4.0	$_{aA}36.2^{a}$ ±1.6	38.6^{a} ±2.2	39.2^{aB} ±4.3	38.6^{a} ±5.5
	N	38.0^{a} ±11.5	$_{aB}46.6^{a}$ ±13.5	46.4^{a} ±10.6	45.6^{a} ±11.2	47.0^{a} ±12.6
240	B	37.0^{a} ±3.8	38.4^{a} ±3.8	37.2^{a} ±2.2	37.0^{a} ±9.8	37.2^{a} ±5.2
	N	45.0^{a} ±7.9	44.4^{a} ±11.4	44.8^{a} ±10.6	43.0^{a} ±16.8	44.2^{a} ±9.5
Overall mean (±SD)	**B**	**$_{a}31.6^{aA}$ ±8.7**	**$_{a}36.7^{aBb}$ ±2.7**	**$_{a}37.4^{aBb}$ ±2.3**	**$_{a}37.4^{aBb}$ ±6.0**	**$_{a}38.2^{b}$ ±4.6**
	N	**$_{b}39.9^{aA}$ ±10.5**	**$_{b}44.7^{ab}$ ±12.3**	**$_{b}45.1^{aBb}$ ±10.6**	**$_{b}45.6^{aBb}$±12.5**	**$_{b}47.5^{b}$ ±11.5**

^{a,b,c} Means in the same row with different superscripts differ significantly ($p<0.05$)

$_{a, b}$ Means in the same cooling and equilibration period with different subscripts differ significantly ($p<0.05$)

$^{aA.aB}$ Means in the same row with different superscripts differ ($p<0.1$)

$_{aA,aB}$ Means in the same cooling and equilibration period with different subscripts differ ($p<0.1$)

^{a,b,c} Means in the same column with different superscripts differ significantly ($p<0.05$)

$^{aA.aB}$ Means in the same column with different superscripts differ ($p<0.1$)

Figure 5.9: The relationship between the cooling period and mean post-thaw sperm motility of cryopreserved semen from Boran bulls

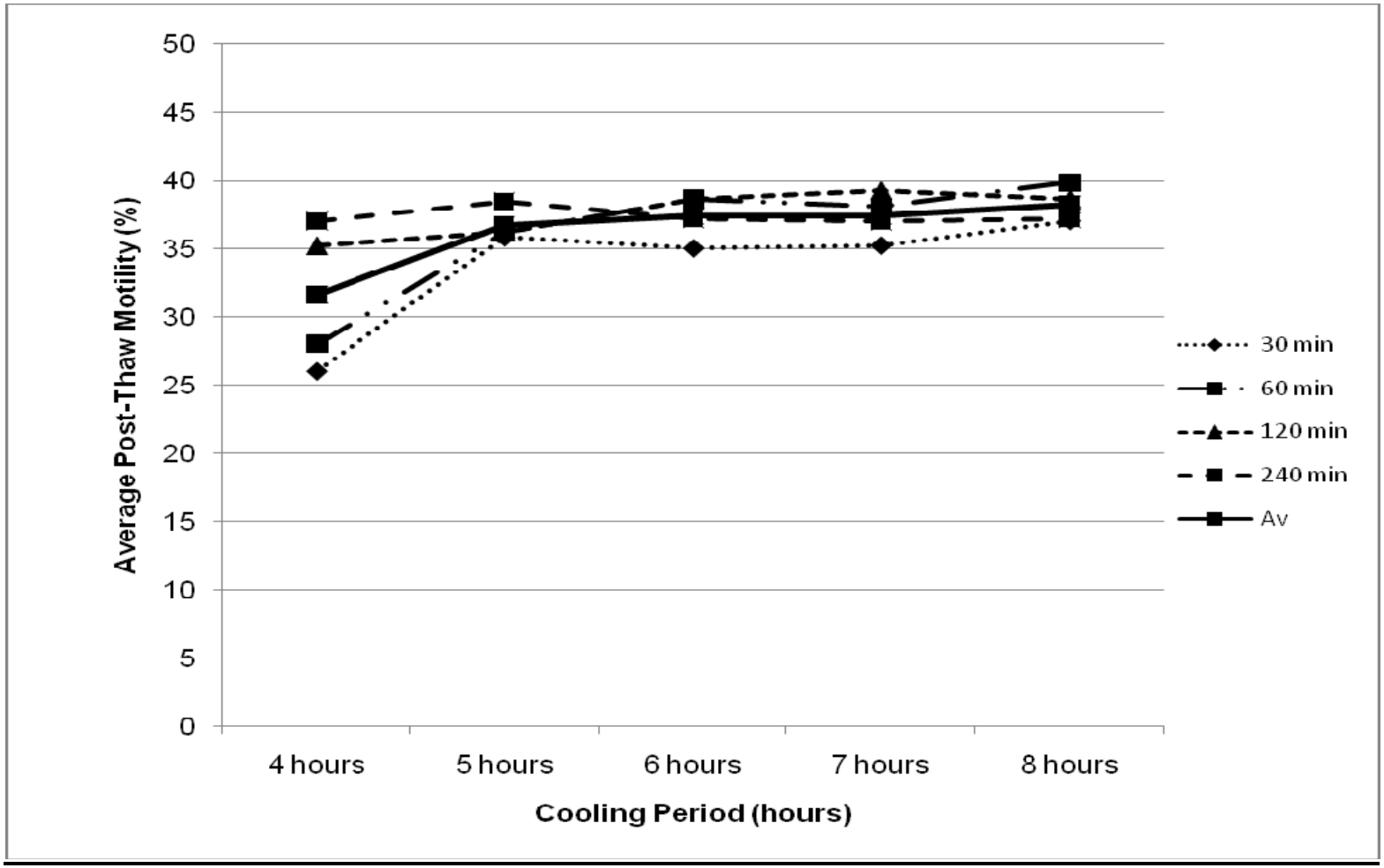
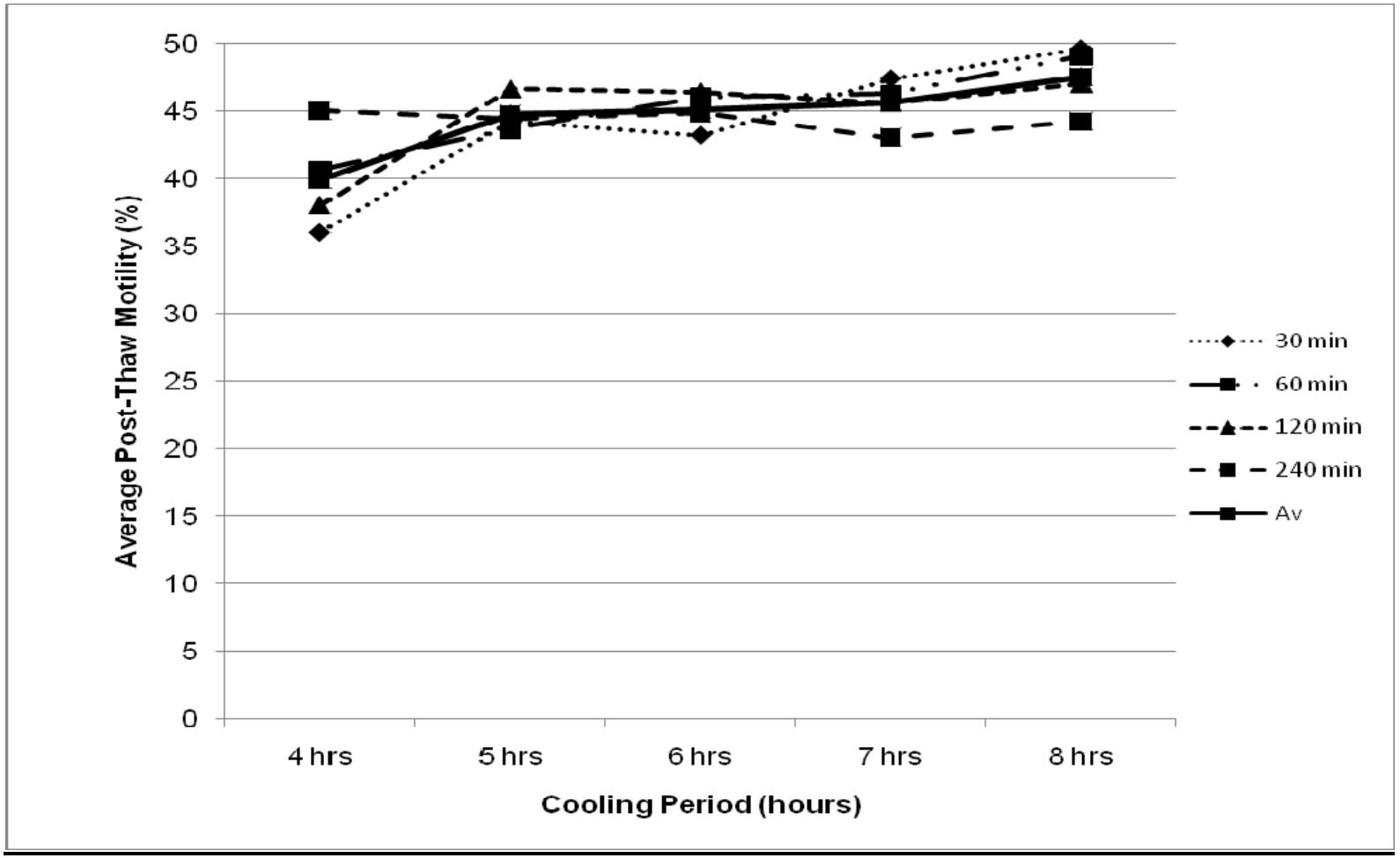


Figure 5.10: The relationship between the cooling period and mean post-thaw sperm motility of cryopreserved semen from Nguni bulls



5.8 Effect of different cooling periods on the recovery fraction of semen from Boran and Nguni bulls

These results of the effect of different cooling periods on the recovery fraction of Boran and Nguni semen are summarized in Table 5.8. The general trend for the effect of different cooling periods on the mean recovery fraction of Boran semen is illustrated in Figure 5.11, while the effect on Nguni semen, is illustrated in Figure 5.12.

The author could not find any previous studies using mean recovery fraction of bovine semen as a variable. The author could not find any previous studies comparing frozen-thawed semen from Boran and Nguni bulls.

5.8.1 Effect of different cooling periods on the recovery fraction of Boran semen

Over all equilibration periods a 4 h cooling period yielded a mean recovery fraction (0.38±0.11) significantly lower ($p<0.05$) than the mean recovery fractions after 6 h (0.45±0.04), 7 h (0.45±0.08) and 8 h (0.46±0.06). The mean recovery fraction resulting from 5 h (0.44±0.04) of cooling tended to differ from the recovery fraction after 4 h. The mean recovery fractions following cooling periods of 5, 6, 7 and 8 h were not significantly different.

With a glycerol equilibration period of 30 minutes, a cooling period of 4 h resulted in the lowest (tended to differ compared to 5, 6 and 7 h and differed $p<0.05$ – from 8 h) the recovery fraction (0.31±0.13), while a period of 8 h gave the highest (not significantly different from 5, 6 and 7 h; differed ($p<0.05$) from 4 h) recovery fraction (0.45±0.06). Periods of 6, 7 and 5 h resulted in increasing mean fractions of 0.42±0.02, 0.42±0.05 and 0.43±0.05, respectively. Cooling for 4 h recorded a significantly lower ($p<0.05$) mean fraction, when compared to the mean recovery fraction after 8 h. There were also a tendency to differ between the Boran recovery fraction after 4 and 5 h, 4 and 6 h, as well as after 4 and 7 h. There were no significant differences between the fractions recorded after 5, 6, 7 and 8 h cooling periods.

With a glycerol equilibration period of 60 minutes, cooling periods of 4, 5, 7, 6 and 8 h yielded increasing mean recovery fractions of 0.34±0.12, 0.44±0.03, 0.46±0.08, 0.47±0.03 and 0.48±0.05. The difference between the recovery fractions after 4 h of glycerol equilibration, was significantly different ($p<0.05$) from that after 8 h of cooling. The recovery fractions after 4 and 6 h, as well as 4 and 7 h of cooling tended to differ from one another. Mean recovery fractions after 5, 6, 7 and 8 h of cooling as well as recovery fractions after 4 and 5 h cooling periods, were not significantly different from one another.

With glycerol equilibration periods of either 120 or 240 minutes, the Boran breed recorded no significant difference regarding the recovery fractions from the different glycerol equilibration periods. Combined with an equilibration period of 120 minutes, cooling semen for 4, 5, 6, 7 and 8 h resulted in mean recovery fractions of 0.42±0.05, 0.44±0.04, 0.47±0.03, 0.47±0.06 and 0.47±0.08.

When a glycerol equilibration period of 240 minutes was used, 5 h of cooling resulted in the highest (no significant difference) mean recovery fraction (0.46±0.05), while cooling periods of 4 h (0.45±0.06), 6 h (0.45±0.04), 7 h (0.45±0.12) and 8 h (0.45±0.07) all resulted in lower (no significant difference) mean recovery fractions.

5.8.2 Effect of different cooling periods on the recovery fraction of Nguni semen

Over all the equilibration periods, a 4 h cooling period recorded an mean recovery fraction (0.46±0.11) significantly lower than fractions after 7 h (0.52±0.13) and 8 h (0.54±0.12) ($p<0.05$). The recovery fraction after 4 h also tended to differ from the mean recovery fractions after 5 h (0.51±0.13) and 6 h (0.52±0.11). Cooling periods of 5, 6, 7 and 8 h resulted in mean recovery fractions that were not significantly different from one another.

A glycerol equilibration period of 30 minutes and a cooling period of 4 h resulted in the lowest (differed significantly from 7 and 8 h; $p<0.05$) recovery fraction (0.41±0.17) while cooling for 8 h yielded the highest (no significant difference for 5, 6 and 7 h) recovery fraction (0.57±0.146). Cooling periods of 6 h (0.50±0.13), 5 h

(0.51±0.15) and 7 h (0.54±0.13) recorded increasing recovery fractions. A cooling period of 4 h resulted in a significantly lower ($p<0.05$) recovery fraction when compared to cooling periods of either 7 or 8 h. There were no significant differences between the mean recovery fractions after cooling periods of 5, 6, 7 and 8 h, as well as after 4 and 5 and also 4 and 6 h.

When a 60 minute glycerol equilibration period was used, longer cooling periods produced higher recovery fractions. The lowest (not significant) mean recovery fraction (0.47±0.07) was the result of a 4 h cooling period, followed by the mean recovery fraction after 5 h (0.50±0.14). Equilibration periods of 6 h (0.53±0.14) and 7 h (0.53±0.13) yielded the second highest (not significant) mean recovery fraction. The highest (also no significant difference) mean recovery fraction (0.56±0.13) was the result of an 8 h period. Differences between mean recovery fractions from the respective equilibration periods were not significantly significant.

With a cooling period of 120 minutes, the lowest (0.43±0.12) and highest (0.54±0.13) mean recovery fractions were the results of 4 and 8 h of equilibration (not significant). The second lowest mean recovery fraction (0.52±0.11) resulted from a 7 h equilibration period, while the second highest mean recovery fraction was the product of either 5 h (0.53±0.14) or 6 h (0.53±0.11) of equilibration (not significant). There were no significant differences between the mean recovery fractions produced by the respective cooling periods.

There were also no significant differences for the Nguni bulls, when a cooling period of 240 minutes was used. Cooling semen for 7 h resulted in the lowest (no significant differences) mean recovery fraction (0.49±0.18), while cooling period of either 4 h (0.52±0.09) or 6 h (0.52±0.11) produced the highest (not significant) recovery fractions. The second highest (no significant differences) mean recovery fraction was the result of either a 5 h (0.51±0.12) or 8 h (0.51±0.10) period of cooling.

5.8.3 Effect of different cooling periods and breed on the recovery fraction of bovine semen

Over all the equilibration periods studied there were significant differences ($p<0.05$) between recovery fractions after all of the cooling periods. So for example the cooling periods of 4, 5, 6, 7 and 8 h resulted in a mean recovery fraction of 0.38±0.11, 0.44±0.04, 0.45±0.04, 0.45±0.08 and 0.46±0.06 for the Boran and 0.46±0.11, 0.51±0.13, 0.52±0.11, 0.52±0.13 and 0.54±0.12 for the Nguni.

When equilibrating diluted semen for 30 minutes after glycerol addition, there were no significant differences between the recovery fractions of the two breeds, after cooling periods of 4, 5 and 6 h. The mean recovery fractions from the two breeds after 7 and 8 h of cooling tended to differ. The Nguni bulls again consistently recorded higher mean recovery fractions – 0.41±0.17, 0.51±0.15, 0.50±0.13, 0.54±0.13 and 0.57±0.15 – after cooling periods of 4, 5, 6, 7 and 8 h, compared to the Boran's mean recovery fractions of 0.31±0.13, 0.43±0.05, 0.42±0.02, 0.42±0.05 and 0.45±0.06 after the same periods of cooling.

When a glycerol equilibration period of 60 minutes was applied for the different cooling periods, there were no significant differences between the recovery fractions for the two breeds after cooling periods of 5, 6, 7 and 8 h. A cooling period of 4 h did result in a significant difference ($p<0.05$) between the mean recovery fractions for the Boran and Nguni bulls. Cooling periods of 4, 5, 6, 7 and 8 h resulted in mean recovery fractions of 0.25±0.19, 0.37±0.15, 0.40±0.09, 0.41±0.15 and 0.42±0.06 for the Boran. The same cooling periods recorded mean recovery fractions of 0.26±0.15, 0.42±0.14, 0.41±0.18, 0.46±0.09 and 0.43±0.16 for the Nguni breed.

With a 120 minute equilibration period, there were no significant differences in the mean recovery fractions after any of the cooling periods. Cooling periods of 4, 5, 6, 7 and 8 h resulted in mean recovery fractions of 0.38±0.11, 0.44±0.08, 0.45±0.05, 0.43±0.03 and 0.43±0.05 for the Boran and 0.38±0.14, 0.41±0.12, 0.52±0.08, 0.50±0.08 and 0.50±0.10 for the Nguni.

After an equilibration period of 240 minutes, no significant differences between the recovery fractions for the two breeds were recorded, when cooling periods of 4, 5, 6, 7 and 8 h were applied. Mean recovery fractions after 4 h of equilibration were

0.43±0.10 for the Boran and 0.49±0.09 for the Nguni. Equilibration for 5 h yielded a mean recovery fraction of 0.42±0.09 (Boran) and 0.52±0.09 (Nguni), while an equilibration period of 6 h resulted in mean recovery fractions of 0.45±0.05 (Boran) and 0.52±0.07 (Nguni). Recovery fractions of 0.47±0.05 (Boran) and 0.52±0.07 (Nguni) were the result of a 7 h equilibration, while the mean recovery fractions of 0.46±0.08 (Boran) and 0.51±0.11 (Nguni) resulted following 8 h of equilibration.

Although there seems to be great variety in the results determined by different researchers over the last few decades, the common theory from these results include a slow cooling rate, as well as a period of equilibration at 5°C for semen prior to the freezing process (Polge, 1957). It was also found that semen equilibrated for 18 h showed greater tolerance when subjected to a slow cooling, compared to semen equilibrated for 1 h.

In a study by Ennen *et al.* (1976) it was found the optimum cooling period for bovine semen to be either 2 or 4 h, combined with a glycerol equilibration period of either 4 to 10 h after a 2 h cooling period, or 2 to 4 h of glycerol equilibration after a 4 h cooling period.

TABLE 5.8: The between and within breed effect of different cooling periods compared within several glycerol equilibration periods on the mean (±SD) recovery fraction of bovine semen

GLYCEROL EQUILIBRATION PERIOD (minutes)	BREED	COOLING PERIOD (h) 4	5	6	7	8
30	B	0.31^{aA} ±0.13	0.43^{aBb} ±0.05	0.42^{aBb} ±0.02	$_{aA}0.42^{aBb}$ ±0.05	$_{aA}0.45^{b}$ ±0.06
	N	0.41^{a} ±0.17	0.51^{ab} ±0.15	0.50^{ab} ±0.13	$_{aB}0.54^{b}$ ±0.13	aB0.57^{b} ±0.15
60	B	$_{a}0.34^{aA}$ ±0.12	0.44^{ab} ±0.03	0.47^{aBb} ±0.03	0.46^{aBb} ±0.08	0.48^{b} ±0.05
	N	$_{b}0.47^{a}$ ±0.07	0.50^{a} ±0.14	0.53^{a} ±0.14	0.53^{a} ±0.13	0.56^{a} ±0.13
120	B	0.42^{a} ±0.05	0.44^{a} ±0.04	0.47^{a} ±0.03	0.47^{a} ±0.06	0.47^{a} ±0.08
	N	0.43^{a} ±0.12	0.53^{a} ±0.14	0.53^{a} ±0.11	0.52^{a} ±0.11	0.54^{a} ±0.13
240	B	0.45^{a} ±0.06	0.46^{a} ±0.05	0.45^{a} ±0.04	0.45^{a} ±0.12	0.45^{a} ±0.07
	N	0.52^{a} ±0.09	0.51^{a} ±0.12	0.52^{a} ±0.11	0.49^{a} ±0.18	0.51^{a} ±0.10
Overall mean (±SD)	**B**	**$_{a}0.38^{aA}$ ±0.11**	**$_{a}0.44^{aBb}$ ±0.04**	**$_{a}0.45^{b}$ ±0.04**	**$_{a}0.45^{b}$ ±0.08**	**$_{a}0.46^{b}$ ±0.06**
	N	**$_{b}0.46^{aA}$ ±0.11**	**$_{b}0.51^{aBb}$ ±0.13**	**$_{b}0.52^{aBb}$ ±0.11**	**$_{b}0.52^{b}$ ±0.13**	**$_{b}0.54^{b}$ ±0.12**

a,b,c Means in the same row with different superscripts differ significantly (p<0.05)
a, b Means in the same cooling and equilibration period with different subscripts differ significantly (p<0.05)
aA.aB Means in the same row with different superscripts differ (p<0.1)
aA,aB Means in the same cooling and equilibration period with different subscripts differ (p<0.1)
a,b,c Means in the same column with different superscripts differ significantly (p<0.05)
aA.aB Means in the same column with different superscripts differ (p<0.1)

Figure 5.11: The relationship between the cooling period and mean post-thaw sperm motility of cryopreserved semen from Boran bulls

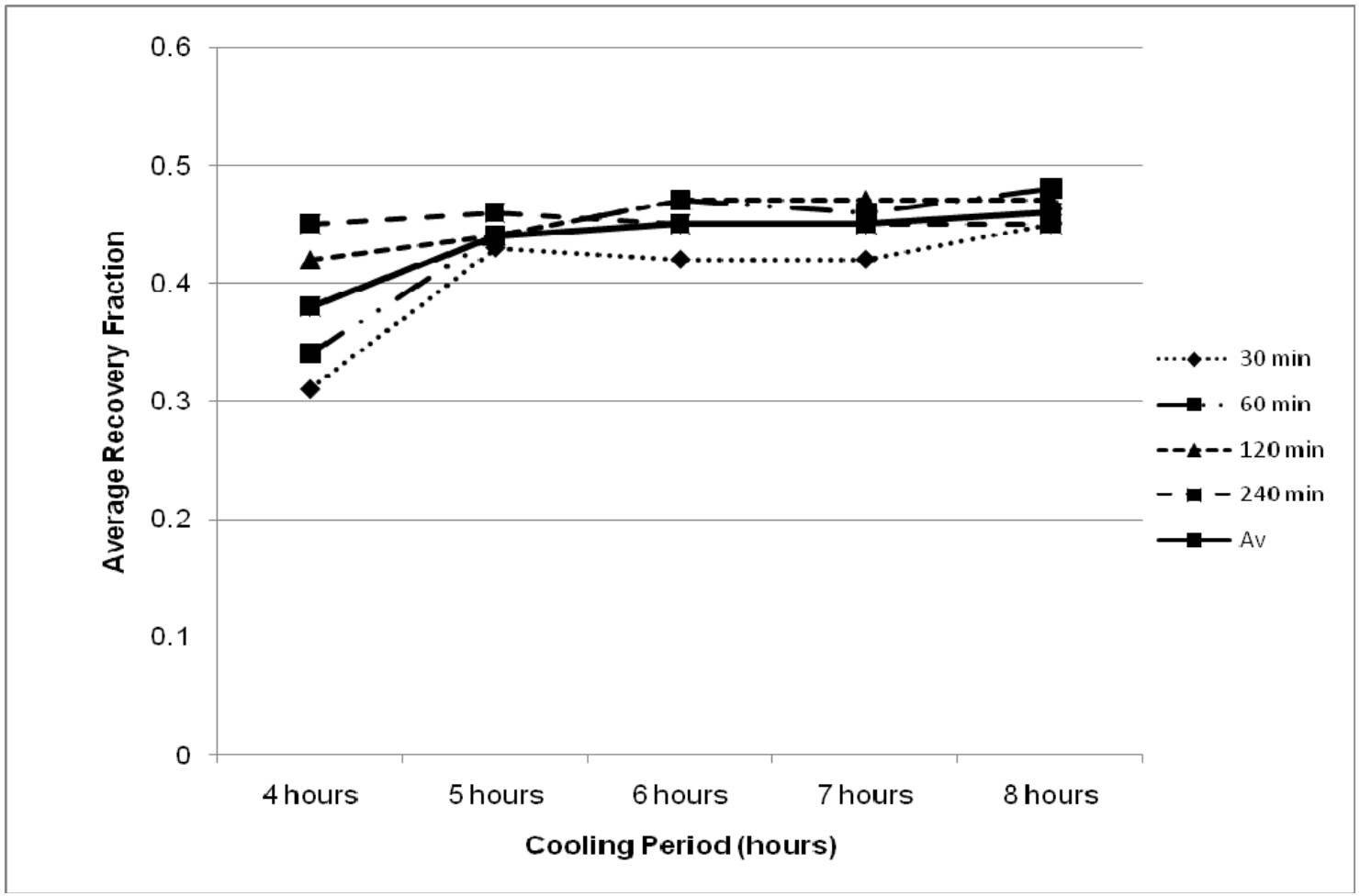
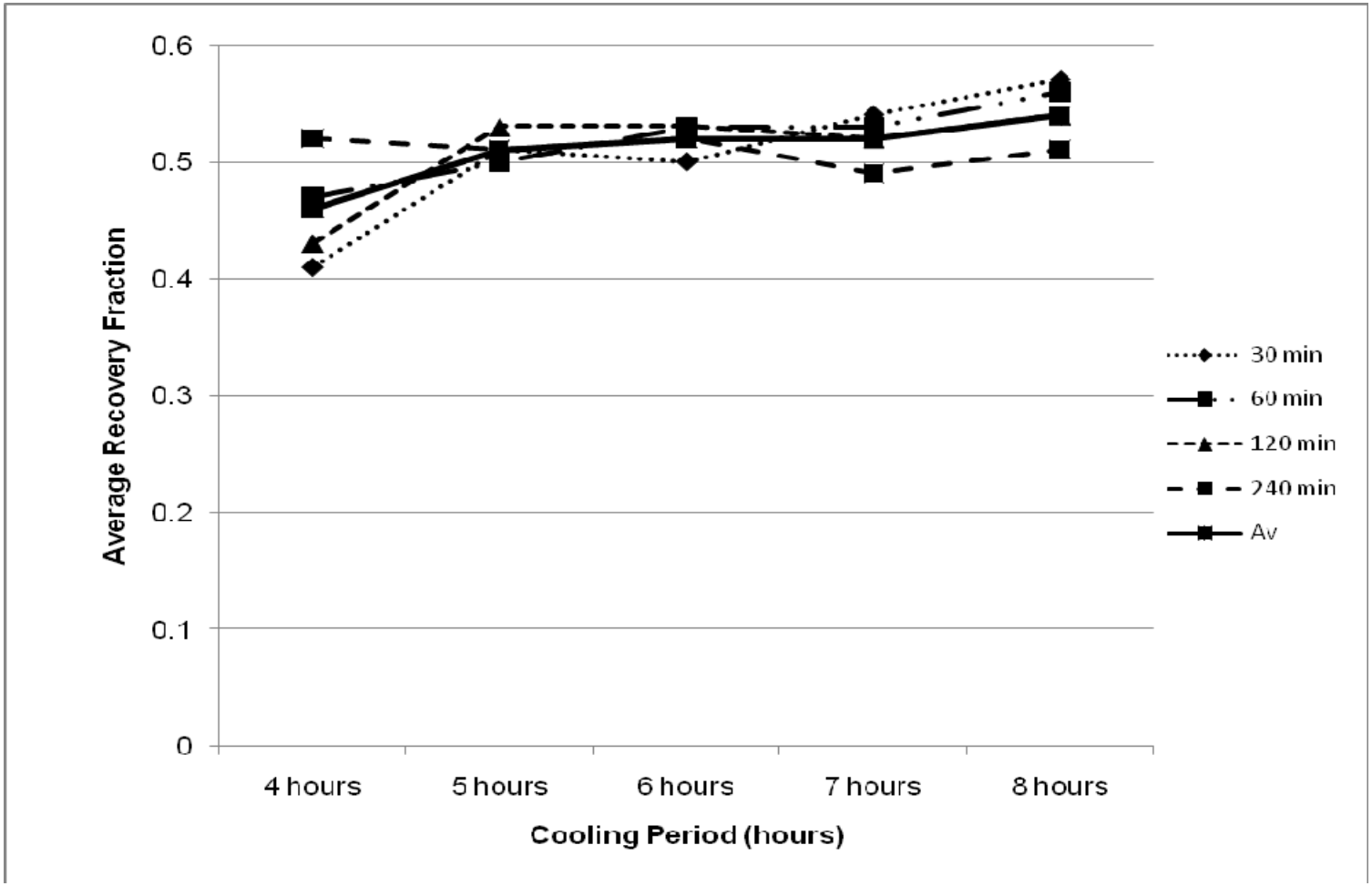


Figure 5.12: The relationship between the cooling period and mean post-thaw sperm motility of cryopreserved semen from Nguni bulls



5.9 Effect of different glycerol equilibration periods on the post-thaw motility of semen from Boran and Nguni bulls

The effects of the different glycerol equilibration periods on the post-thaw sperm motility rate are summarized in Table 5.9. The general trend regarding the effect of different glycerol equilibration periods on the mean post-thaw motility rate of Boran semen is illustrated in Figure 5.13, together with that of the Nguni semen also illustrated in Figure 5.14.

The author could not find any previous studies comparing frozen-thawed semen from Boran and Nguni bulls.

5.9.1 Effect of different glycerol equilibration periods on the post-thaw sperm motility for Boran semen

Over all the cooling periods, a 30 minute period produced the lowest (not significant) mean post-thaw sperm motility (33.8±6.7%) for the Boran. Equilibration periods of 60 (36.1±6.7%), 240 (37.4±5.1%) and 120 (37.6±3.8%) minutes resulted in increasing mean post-thaw motility rates. The differences between the post-thaw motility rates for the different equilibration periods were not significantly different.

Graham *et al.* (1956) found no significant difference ($p<0.05$) between the non-return rate in cows inseminated with semen that was equilibrated with glycerol for either 4 or 8 h.

Similarly it was reported that a 2 h glycerol equilibration period yielded significantly higher ($p<0.01$) post-thaw motility rates than a 1 h glycerol equilibration period (Dhami and Sahni, 1993). This is not in agreement with the results obtained from the present study, which found no significant difference between the Boran post-thaw sperm motility, yielded by either a 1 or a 2 h cooling period.

Martin and Emmens (1961) reported the post-thaw motility after a 30 minute glycerol equilibration period to be significantly lower ($p<0.001$) than after an 18 h glycerol equilibration period. In the present study there was no significant difference between

the Boran post-thaw motility, yielded after glycerol equilibration periods of either 30 minutes or longer periods of 60, 120 or 240 minutes.

When comparing the different glycerol equilibration periods following 4 h of cooling, there was a tendency to differ when comparing the mean Boran post-thaw motility rates for 30 and 240 minutes of equilibration. The post-thaw sperm motility increased, as the period of equilibration lengthened. Periods of 30, 60, 120 and 240 minutes yielded mean post-thaw motility of 26.0±10.8%, 28.0±10.4%, 35.2±4.0% and 37.0±3.8%. Mean post-thaw motility rates resulting following periods of 60, 120 and 240 minutes were not significantly different. When comparing the post-thaw motility rates for periods of 30 and 60, as well as 30 and 120 minutes, no significant difference was recorded in the difference between these post-thaw motility rates.

When 5 h of cooling followed the various glycerol equilibration periods, the post-thaw motility rates increased (not significantly different), when equilibration periods of 30 (35.8±3.1%), 60 (36.2±1.6%), 120 (36.2±1.6%) and 240 minutes (38.4±3.8%) were arranged, in that order. The differences in mean post-thaw motility rates, after the different equilibration periods were not significant.

When the various glycerol equilibration periods followed a cooling period of 6 h, the differences between the respective post-thaw motility rates of the Boran were not significant. An equilibration period of 30 minutes produced the lowest (no significant difference) mean post-thaw motility (35.0±0.0%) followed by a period of 240 (37.2±2.2%), while periods of 60 (38.6±2.2%) and 120 minutes (38.6±2.2%) resulted in the highest (also no significant difference) post-thaw motility.

With 7 h of cooling, equilibration periods of 30, 240, 60 and 120 minutes produced increasing post-thaw motility rates of 35.2±4.0%, 37.0±9.8%, 38.0±5.7% and 39.2±4.3%. These differences between the post-thaw motility rates after different equilibration periods, were not significant.

When combining glycerol equilibration periods of 30 (37.0±5.1%), 240 (37.2±5.2%), 120 (38.6±5.5%) and 60 minutes (39.8±3.0%) with an 8 h cooling period, progressively higher mean post-thaw motility rates were recorded. There were no significant differences between the post-thaw motility resulting from the respective cooling periods.

5.9.2 Effect of different glycerol equilibration periods on the post-thaw sperm motility of Nguni semen

For the Nguni a 30 minute glycerol equilibration period produced the lowest post-thaw motility rate (44.1±13.3%) when taken over all the cooling periods. An increase in the post-thaw motility resulting from periods of 240 (44.3±10.6%), 120 (44.7±11.4%) and 60 (45.1±11.4%) minutes. None of the differences between these mean motility rates were significant.

Martin and Emmens (1961) reported the post-thaw sperm motility after a 30 minute glycerol equilibration period to be significantly lower ($p<0.001$), than after an 18 h glycerol equilibration period. In the present study there was no significance ($p<0.05$ or $p<0.1$) in the differences between Nguni mean post-thaw motility rates recorded after glycerol equilibration periods of either 30minutes, or longer periods of 60, 120 or 240 minutes.

A 2 h glycerol equilibration period yielded significantly higher ($p<0.01$) post-thaw motility, compared to a 1 h glycerol equilibration period (Dhami and Sahni, 1993). This is not in agreement with the results of the present study, which found no significant difference between the mean Nguni post-thaw motility yielded by either a 1 or a 2 h cooling period. Almlid and Johnson (1988) studied the effects of glycerol equilibration periods on boar semen, and established no significant differences in the motility of spermatozoa equilibrated for 0.5, 2, 5, 15 or 75 minutes. Leite *et al.* (2010) reported that glycerol equilibration periods of either 2 or 4 h resulted in significantly higher motility than a no glycerol equilibration period. Tuli *et al.*(1981) determined that a 4 h glycerol equilibration period recorded superior post-thaw motility rates when compared to glycerol equilibration periods of either 2 of 6 h.

When comparing the different glycerol equilibration periods following 4 h of cooling, a 30 minute period resulted in the lowest post-thaw motility rate (36.0±15.2%). When using periods of 120 (38.0±11.5%), 60 (40.6±6.67%) and 240 minutes (45.0±7.9%), the mean post-thaw motility rate progressively increased (although not significant). The Nguni breed recorded no significant differences in the mean post-thaw motility rates for the different cooling periods.

When 5 h of cooling, followed by glycerol equilibration periods of 120 (46.6±13.5%), 240 (44.4±11.4%), 30 (44.2±14.3%) and 60 (43.6±13.9%) minutes, the mean post-thaw motility rate declined. There were no significant differences between mean post-thaw motility rates after the different equilibration periods.

With 6 h of cooling, glycerol equilibration periods of 30 (43.2±11.6%), 240 (44.8±10.6%), 60 (46.0±12.9%) and 120 (46.4±10.6%) minutes recorded post-thaw sperm motility rates that increased (not significant). The Nguni recorded no significant differences between the rates following the different equilibration periods.

When a cooling period of 7 h followed by glycerol equilibration periods of 240 (43.0±16.8%), 120 (45.6±11.2%), 60 (46.2±13.0%) or 30 (47.4±12.5%) minutes, post-thaw motility rates increased (not significantly different). There were no significant differences between the post-thaw motility rates after the different equilibration periods.

Equilibration periods of 30 (49.6±14.1%), 60 (49.0±12.4%), 120 (47.0±12.6%) and 240 minutes (44.2±9.5%) following an 8 h cooling period, produced post-thaw motility rates that were not significantly different. Mean post-thaw motility rates decreased, with longer glycerol equilibration periods.

5.9.3 Effects of different glycerol equilibration periods and breed on the difference between post-thaw sperm motility of bovine semen

Comparing the mean post-thaw motility rates of the two breeds over all the cooling periods, demonstrated significant differences ($p<0.05$) for all four equilibration periods. Periods of 30, 60, 120 and 240 minutes resulted in mean post-thaw motility rates of 33.8±6.7%, 36.1±6.7%, 37.6±3.8% and 37.4±5.1% for the Boran as well as 44.1±13.3%, 45.1±11.4%, 44.7±11.4% and 44.3±10.6% for the Nguni bulls.

When comparing the post-thaw sperm motility of the two breeds after a cooling period of 4 h, followed by the respective equilibration periods, there was a significant difference ($p<0.05$) between the post-thaw motility of the two breeds, with a 60 minute equilibration period. There was also a tendency towards significant difference between the mean post-thaw motility rates for the two breeds with 30 minutes of

equilibration. No significant differences were recorded between the mean post-thaw motility for the two breeds, when semen was equilibrated for 120 or 240 minutes. Glycerol equilibration periods of 30, 60, 120 and 240 minutes resulted in post-thaw motility rate of 26.0±10.8%, 28.0±10.4%, 35.2±4.0% and 37.0±3.8% for the Boran breed. The Nguni bulls yielded higher mean post-thaw motility rates of 36.0±15.2%, 40.6±6.7%, 38.0±11.5% and 45.0±7.9% following the corresponding equilibration periods.

After a cooling period of 5 h, there were no significant difference when comparing the post-thaw motility of the two breeds, after equilibration periods of 30, 60 or 240 minutes. Equilibrating semen for 120 minutes resulted in mean post-thaw motility rates that tended to differ for the Boran and Nguni. After glycerol equilibration periods of 30, 60, 120 and 240 minutes the Boran recorded post-thaw motility rates of 35.8±3.1%, 36.2±1.6%, 36.2±1.6% and 38.4±3.8% respectively, while the Nguni yielded mean post-thaw motility rates of 44.2±14.3%, 43.6±13.9%, 46.6±13.5% and 44.4±11.4%.

When partly diluted semen was allowed to cool for 6 h prior to glycerol addition, and then left for different equilibration periods, no significant differences were recorded between the resulting post-thaw sperm motility rates of the two breeds. The Boran recorded post-thaw motility rates of 35.0±0.0%, 38.6±2.2%, 38.6±2.2% and 37.2±2.2% after glycerol equilibration periods of 30, 60,120 and 240 minutes. The Nguni bulls again yielded post-thaw motility rates of 43.2±11.6%, 46.0±12.9%, 46.4±10.6% and 44.8±10.6%, after the same periods of glycerol equilibration.

Cooling semen for 7 h, prior to glycerol addition and then allowing semen to equilibrate for periods of 60, 120 and 240 minutes did not result in any significant differences between the post-thaw motility rates of the Boran and Nguni bulls. There was however a significant difference ($p<0.05$) between the resulting post-thaw sperm motility of the two breeds, with an equilibration period of 30 minutes. After equilibration periods of 30, 60, 120 or 240 minutes the Boran yielded a mean post-thaw motility of 35.2±4.0%, 38.0±5.7%, 39.2±4.3% and 37.0±9.8%. The same equilibration periods resulted in mean post-thaw motility rates of 47.4±12.5%, 46.2±13.0%, 45.6±11.2% and 43.0±16.8% for the Nguni breed.

There was a significant difference ($p<0.05$) between the mean post-thaw motility rates of the Boran and Nguni using an 8 h cooling and 30 minute equilibration period. Cooling semen for 8 h, combined with glycerol equilibration periods of 60, 120 or 240 minutes resulted in mean post-thaw motility rates that did not significantly differ between the two breeds. Equilibrating semen for 30, 60, 120 or 240 minutes resulted in mean rates of 37.0±5.1%, 39.8±3.0%, 38.6±5.5% and 37.2±5.2% for the Boran, as well as 49.6±14.1%, 49.0±12.4%, 47.0±12.6% and 44.2±9.5% for the Nguni.

TABLE 5.9: The between and within breed effects of different glycerol equilibration periods compared within several cooling periods on the mean (±SD) post-thaw sperm motility of bovine semen

COOLING PERIOD (h)	BREED	GLYCEROL EQUILIBRATION PERIOD (minutes) 30	60	120	240
4	B	$_{aA}26.0^{aA}$ ±10.8	$_{a}28.0^{a}$ ±10.4	35.2^{a} ±4.0	37.0^{aB} ±3.8
	N	$_{aB}36.0^{a}$ ±15.2	$_{b}40.6^{a}$ ±6.7	38.0^{a} ±11.5	45.0^{a} ±7.9
5	B	35.8^{a} ±3.1	36.2^{a} ±1.6	$_{aA}36.2^{a}$ ±1.6	38.4^{a} ±3.8
	N	44.2^{a} ±14.3	43.6^{a} ±13.9	$_{aB}46.6^{a}$ ±13.5	44.4^{a} ±11.4
6	B	35.0^{a} ±0.0	38.6^{a} ±2.2	38.6^{a} ±2.2	37.2^{a} ±2.2
	N	43.2^{a} ±11.6	46.0^{a} ±12.9	46.4^{a} ±10.6	44.8^{a} ±10.6
7	B	$_{a}35.2^{a}$ ±4.0	38.0^{a} ±5.7	39.2^{a} ±4.3	37.0^{a} ±9.8
	N	$_{b}47.4^{a}$ ±12.5	46.2^{a} ±13.0	45.6^{a} ±11.2	43.0^{a} ±16.8
8	B	$_{a}37.0^{a}$ ±5.1	39.8^{a} ±3.0	38.6^{a} ±5.5	37.2^{a} ±5.2
	N	$_{b}49.6^{a}$ ±14.1	49.0^{a} ±12.4	47.0^{a} ±12.6	44.2^{a} ±9.5
Overall mean (±SD)	**B**	**$_{a}33.8^{a}$ ±6.7**	**$_{a}36.1^{a}$ ±6.7**	**$_{a}37.6^{a}$ ±3.8**	**$_{a}37.4^{a}$ ±5.1**
	N	**$_{b}44.1^{a}$ ±13.3**	**$_{b}45.1^{a}$ ±11.4**	**$_{b}44.7^{a}$ ±11.4**	**$_{b}44.3^{a}$ ±10.6**

$_{a,b,c}$ Means in the same row with different superscripts differ significantly ($p<0.05$)

$_{a, b}$ Means in the same cooling and equilibration period with different subscripts differ significantly ($p<0.05$)

$^{aA.aB}$ Means in the same row with different superscripts tend to differ ($p<0.1$)

$_{aA,aB}$ Means in the same cooling and equilibration period with different subscripts tend to differ ($p<0.1$)

$^{a.b.c}$ Means in the same column with different superscripts differ significantly ($p<0.05$)

$^{aA.aB}$ Means in the same column with different superscripts tend to differ ($p<0.1$)

Figure 5.13: The relationship between glycerol equilibration period and mean post-thaw sperm motility of cryopreserved semen from Boran bulls

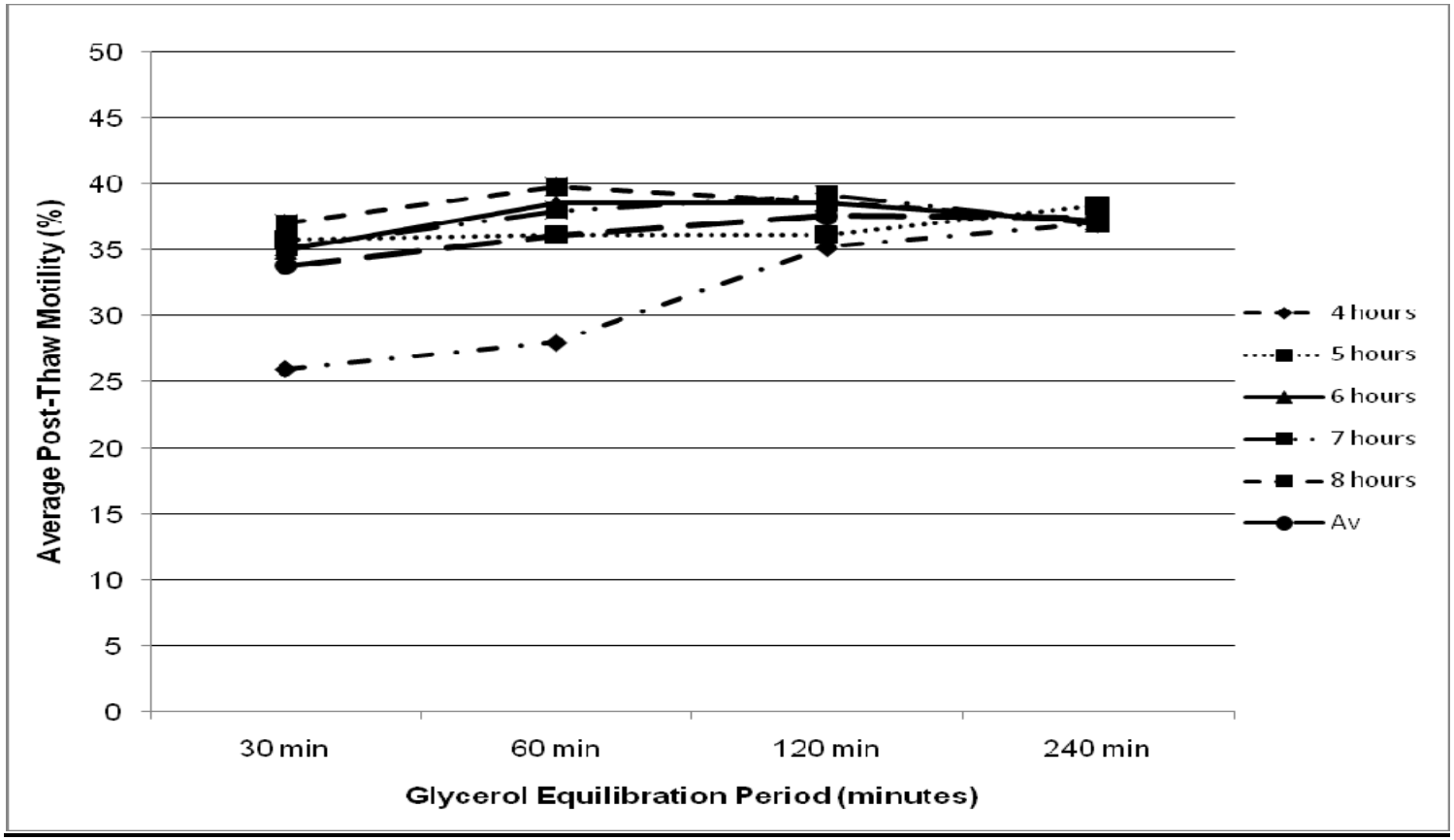
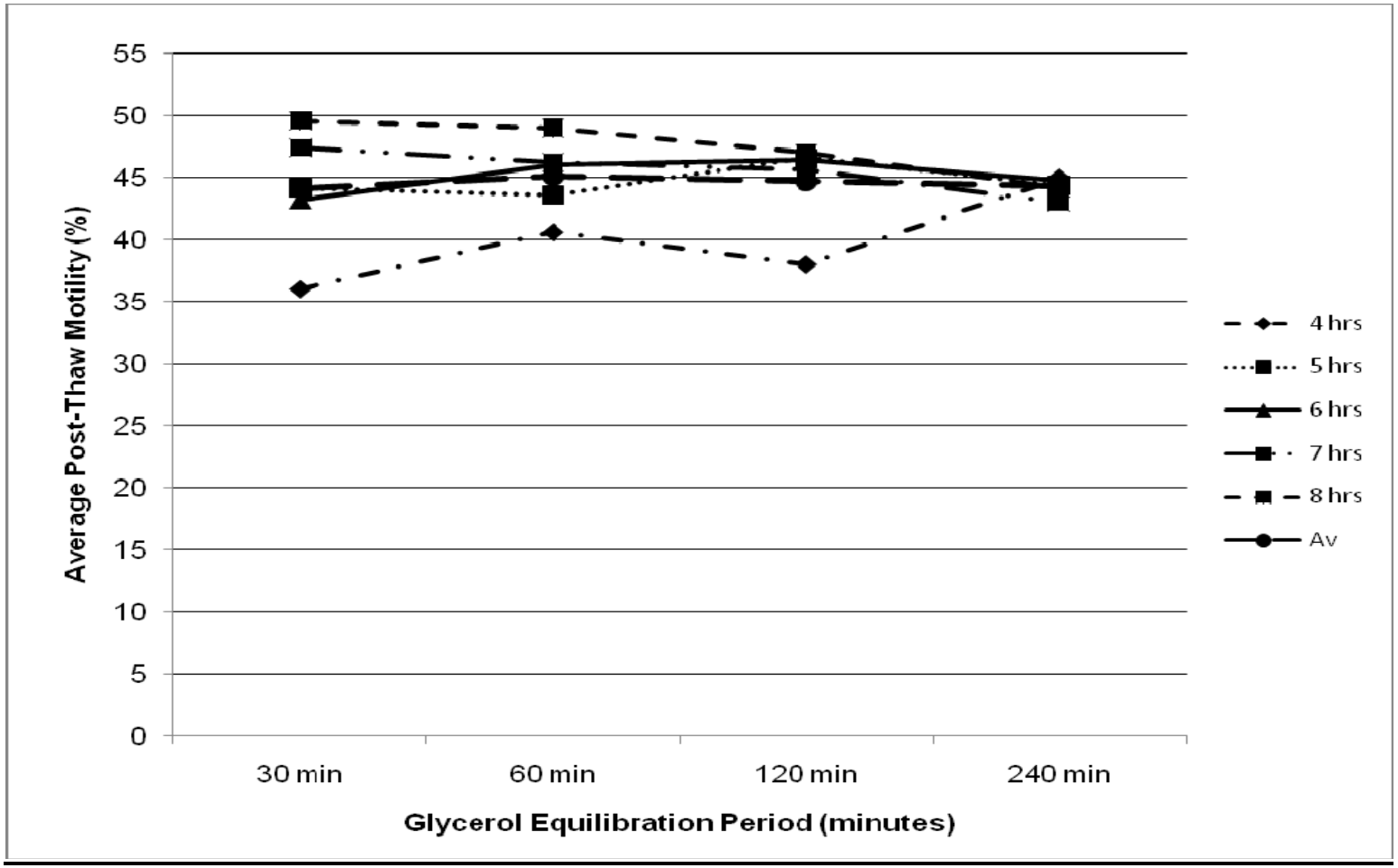


Figure 5.14: The relationship between glycerol equilibration period and mean post-thaw sperm motility of cryopreserved semen from Nguni bulls



5.10 Effect of different glycerol equilibration periods on the recovery fractions of semen from Boran and Nguni bulls

The effect of different glycerol equilibration periods on the recovery fractions of Boran and Nguni bulls are summarized in Table 5.10. The general trend regarding the effect of different glycerol equilibration periods on the recovery fraction of Boran semen is illustrated in Figure 5.15, together with the effect on Nguni semen is illustrated in Figure 5.16.

The author could not find any previous studies using mean recovery fraction of bovine semen as a variable. The author could not find any previous studies comparing frozen-thawed semen from Boran and Nguni bulls.

5.10.1. Effect of different glycerol equilibration periods on the recovery fractions of Boran semen

Overall there were no significant differences when comparing the overall mean recovery fractions for the different equilibration periods irrespective of cooling period. As equilibration periods increased, the resulting mean recovery fraction also increased. Periods of 30, 60, 120 and 240 minutes produced mean recovery fractions of 0.41±0.08, 0.44±0.08, 0.45±0.05 and 0.45±0.07, respectively.

When comparing the different glycerol equilibration periods following 4 h of cooling, there were significant differences ($p<0.05$) between the recovery fractions after 30 and 240 minutes. The Boran recovery fractions following cooling periods of 30 and 120 minutes, as well as from 60 and 240 minutes of cooling, were not significantly different. There were also no significant differences between the recovery fractions from glycerol equilibration periods for 30 and 60, 60 and 120, as well as 120 and 240 minutes. Mean recovery fractions increased as cooling period lengthened. Thus, a period of 30 minutes produced the lowest (not significant from 60 minutes and tended to differ from 120 minutes, while being significantly different ($p<0.05$) from 240 minutes) fraction (0.31±0.13) followed by periods of 60 (0.34±0.12), 120 (0.42±0.05) and 240 minutes (0.45±0.06).

Equilibration periods of 30 and 240 minutes resulted in the lowest (not significant) (0.43±0.05) and highest (0.46±0.05) recovery fractions when combined with 5 h of cooling. Periods of 60 and 120 minutes both recorded the second highest (0.44±0.03 and 0.44±0.04) recovery fraction. The differences in mean recovery fractions for the different equilibration periods were also not significantly significant.

When the various glycerol equilibration periods were followed by a cooling period of 6 h, both the 60 and 120 minutes of cooling produced the highest (not significant) mean recovery fraction (0.47±0.03). A period of 30 minutes again resulted in the lowest mean recovery fraction (0.42±0.02), while a 240 minute period yielded the second lowest (0.45±0.04). These differences between the respective fractions for the Boran were not significant.

With 7 h of cooling, glycerol equilibration periods of 30 (0.42±0.05), 240 (0.45±0.12), 60 (0.46±0.08) and 120 minutes (0.47±0.06) resulted in increasing (not significant) mean recovery fractions. The Boran recorded no significant differences between any of the recovery fractions.

When an 8 h cooling period was followed by equilibration periods of 30, 240, 120 and 60 minutes, the mean recovery fractions increased – but the differences between the respective mean fractions were not significantly significant. The lowest (not significantly different) mean recovery fraction (0.45±0.06) was the result of a 30 minute cooling period, while 60 minutes of cooling resulted in the highest (significantly different) mean recovery fraction (0.48±0.05). Periods of 240 and 120 minutes recorded mean recovery fractions of 0.45±0.07 and 0.47±0.08.

5.10.2. Effects of different glycerol equilibration periods on the recovery fractions of Nguni semen

There were no significant differences between the overall recovery fractions for the respective glycerol equilibration times. Increasing recovery fractions resulted from equilibration periods of 30 (0.50±0.14), 120 (0.51±0.12) and 240 (0.51±0.11) minutes while equilibration for 60 minutes produced the highest (not significant) recovery fraction (0.52±0.12).

When comparing the different glycerol equilibration periods following 4 h of cooling, periods of 240 and 30 minutes yielded the highest (not significantly different from 60 or 120 minutes, but tended to differ from 30 minutes) (0.52±0.09) and lowest (not significantly different from 60 or 120 minutes, but tended to differ from 240 minutes) (0.41±0.17) mean recovery fractions. Equilibration for 60 and 120 minutes produced the second highest (0.47±0.07) and second lowest (0.43±0.12) recovery fractions. The Nguni mean recovery fractions resulting from glycerol equilibration periods of 30 and 240 minutes tended to differ. Recovery fractions following equilibration periods of 60, 120 or 240 minutes were not significantly different from one another. There were also no significant differences between the mean fractions from equilibrating semen for 30 and 60 and also 30 and 120 minutes.

When 5 h of cooling was followed by the various equilibration periods, the Nguni recorded a smaller (not significant) recovery fraction after 60 minutes (0.50±0.14) compared to equilibration periods of 30 (0.51±0.15), 240 (0.51±0.12) and 120 (0.53±0.14) minutes, that produced progressively higher mean recovery fractions. These differences between the respective recovery fractions were not significantly different.

The combination of a 30 minute glycerol equilibration period and 6 h of cooling resulted in the lowest mean recovery fraction (0.50±0.13). When the equilibration period was increased to 240 minutes, the resulting recovery fraction was the second highest (0.52±0.11). Equilibration periods of 60 and 120 minutes recorded the highest mean recovery fractions of 0.53±0.14 and 0.53±0.11. There were no significant differences between the respective mean recovery fractions from different glycerol equilibration periods.

When glycerol equilibration periods of 30, 60, 120 and 240 minutes were followed by a cooling period of 7 h, the resulting recovery fractions were 0.54±0.13, 0.53±0.13, 0.52±0.11 and 0.49±0.18. The mean recovery fractions decreased, as cooling periods increased. There were no significant differences between fractions from the respective cooling periods.

With 8 h of cooling, the Nguni recorded no significant differences for the fractions after 30, 60, 120 and 240 minutes of glycerol equilibration. Mean recovery fraction

steadily decreased, when the length of the cooling period increased. The lowest mean recovery fraction (0.51±0.10) was the result of a 240 minute cooling period, while the highest mean recovery fraction (0.57±0.15) was obtained when using a 30 minute cooling period. Periods of 60 and 120 minutes produced mean recovery fractions of 0.56±0.13 and 0.54±0.13 each.

5.10.3. Effect of different glycerol equilibration periods and breed on the recovery fractions of bovine semen

When equilibration periods between the breeds were compared over all cooling periods, there were significant differences ($p<0.05$) between the mean recovery fractions after 30, 60 or 120 minutes of equilibration. The mean recovery fractions were 0.41±0.08, 0.44±0.08 and 0.45±0.05 for the Boran, while the Nguni recorded mean recovery fractions of 0.50±0.14, 0.52±0.12 and 0.51±0.12. Equilibrating semen for 240 minutes after cooling, resulted in a tendency to differ between the mean recovery fractions of the Boran (0.45±0.07) and Nguni (0.51±0.11) bulls.

There were no significant differences between the recovery fractions for the different breeds, when combining a cooling period of 4 h and glycerol equilibration periods of 30, 120 or 240 minutes. Combining a 4 h cooling period with 60 minutes of equilibration, did result in a significant difference ($p<0.05$) between the mean recovery fractions for the two breeds. An equilibration period of 30 minutes yielded a mean recovery fraction of 0.31±0.13 for the Boran and 0.41±0.17 for the Nguni, while extending semen for 60 minutes resulted in mean recovery fractions of 0.34±0.12 and0.47±0.07. Mean recovery fractions of 0.42±0.05 and 0.45±0.06 (Boran), as well as 0.43±0.12 and 0.52±0.09 (Nguni) were the results of allowing semen to cool for 120 or 240 minutes, respectively.

A cooling period of 5 h, combined with the various equilibration periods yielded mean recovery fractions that were not significantly different between the two breeds. Cooling semen for 30, 60, 120 and 240 minutes resulted in mean recovery fractions of 0.43±0.05, 0.44±0.03, 0.44±0.04 and 0.46±0.05 for the Boran, while the same periods of cooling yielded recovery fractions of 0.51±0.15, 0.50±0.14, 0.53±0.14 and 0.51±0.12 for the Nguni.

Combining equilibration periods of 30, 60, 120 or 240 minutes with 6 h of cooling, recorded no significant differences between resulting recovery fractions of the two breeds. The mean recovery fractions resulting from 30 minutes of cooling were 0.42±0.02 (Boran) and 0.50±0.13 (Nguni), while mean recovery fractions after 60, 120 and 240 minute cooling periods were 0.47±0.03, 0.47±0.03 and 0.45±0.04 for the Boran, as well as 0.53±0.14, 0.53±0.11 and 0.52±0.11 for the Nguni.

With a 7 h cooling period followed by glycerol equilibration periods of 60, 120 or 240 minutes, there were no significant differences between the recovery fractions of the two breeds. A 30 minute equilibration period did however result in a tendency towards significant difference, between the breed recovery fractions. Mean recovery fractions after 30 minutes of equilibration were 0.42±0.05 and 0.54±0.13 for the Boran and Nguni bulls, respectively. An equilibration period of 60 minutes resulted in mean recovery fractions of 0.46±0.08 (Boran) and 0.53±0.13 (Nguni). Equilibration periods of 120 or 240 minutes resulted in mean recovery fractions of 0.47±0.06 and 0.45±0.12 for the Boran, as well as 0.52±0.11 and 0.49±0.18 for the Nguni.

When combining 8 h of cooling with an equilibration period of 30 minutes; the mean recovery fractions for the Boran and Nguni tended to differ. Equilibration periods of 60, 120 or 240 minutes resulted in no significant differences between the recovery fractions of the breeds. Equilibrating semen for periods of 30, 60, 120 or 240 minutes yielded mean recovery fractions of 0.45±0.06, 0.48±0.05, 0.47±0.08 and 0.45±0.07 for the Boran, and the Nguni recorded recovery fractions of 0.57±0.15, 0.56±0.13, 0.54±0.13 and 0.51±0.10.

TABLE 5.10: The between and within breed effects of different glycerol equilibration periods compared within several cooling periods on the mean (±SD) recovery fraction of bovine semen

COOLING PERIOD (h)	BREED	GLYCEROL EQUILIBRATION PERIOD (minutes) 30	60	120	240
4	B	$0.31^{aA} \pm 0.13$	${}_{a}0.34^{abA} \pm 0.12$	$0.42^{aBb} \pm 0.05$	$0.45^{bB} \pm 0.06$
	N	$0.41^{aA} \pm 0.17$	${}_{b}0.47^{a} \pm 0.07$	$0.43^{a} \pm 0.12$	$0.52^{aB} \pm 0.09$
5	B	$0.43^{a} \pm 0.05$	$0.44^{a} \pm 0.03$	$0.44^{a} \pm 0.04$	$0.46^{a} \pm 0.05$
	N	$0.51^{a} \pm 0.15$	$0.50^{a} \pm 0.14$	$0.53^{a} \pm 0.14$	$0.51^{a} \pm 0.12$
6	B	$0.42^{a} \pm 0.02$	$0.47^{a} \pm 0.03$	$0.47^{a} \pm 0.03$	$0.45^{a} \pm 0.04$
	N	$0.50^{a} \pm 0.13$	$0.53^{a} \pm 0.14$	$0.53^{a} \pm 0.11$	$0.52^{a} \pm 0.11$
7	B	${}_{aA}0.42^{a} \pm 0.05$	$0.46^{a} \pm 0.08$	$0.47^{a} \pm 0.06$	$0.45^{a} \pm 0.12$
	N	${}_{aB}0.54^{a} \pm 0.13$	$0.53^{a} \pm 0.13$	$0.52^{a} \pm 0.11$	$0.49^{a} \pm 0.18$
8	B	${}_{aA}0.45^{a} \pm 0.06$	$0.48^{a} \pm 0.05$	$0.47^{a} \pm 0.08$	$0.45^{a} \pm 0.07$
	N	${}_{aB}0.57^{a} \pm 0.15$	$0.56^{a} \pm 0.13$	$0.54^{a} \pm 0.13$	$0.51^{a} \pm 0.10$
Overall mean (±SD)	**B**	**${}_{a}0.41^{a} \pm 0.08$**	**${}_{a}0.44^{a} \pm 0.08$**	**${}_{a}0.45^{a} \pm 0.05$**	**${}_{aA}0.45^{a} \pm 0.07$**
	N	**${}_{b}0.50^{a} \pm 0.14$**	**${}_{b}0.52^{a} \pm 0.12$**	**${}_{b}0.51^{a} \pm 0.12$**	**${}_{aB}0.51^{a} \pm 0.11$**

a,b,c Means in the same row with different superscripts differ significantly (p<0.05)
a, b Means in the same cooling and equilibration period with different subscripts differ significantly (p<0.05)
aA.Ab Means in the same row with different superscripts tend to differ (p<0.1)
aA,aB Means in the same cooling and equilibration period with different subscripts tend to differ (p<0.1)
a,b,c Means in the same column with different superscripts differ significantly (p<0.05)
aA.aB Means in the same column with different superscripts tend to differ (p<0.1)

Figure 5.15: The relationship between glycerol equilibration period and mean recovery fraction of cryopreserved semen from Boran bulls

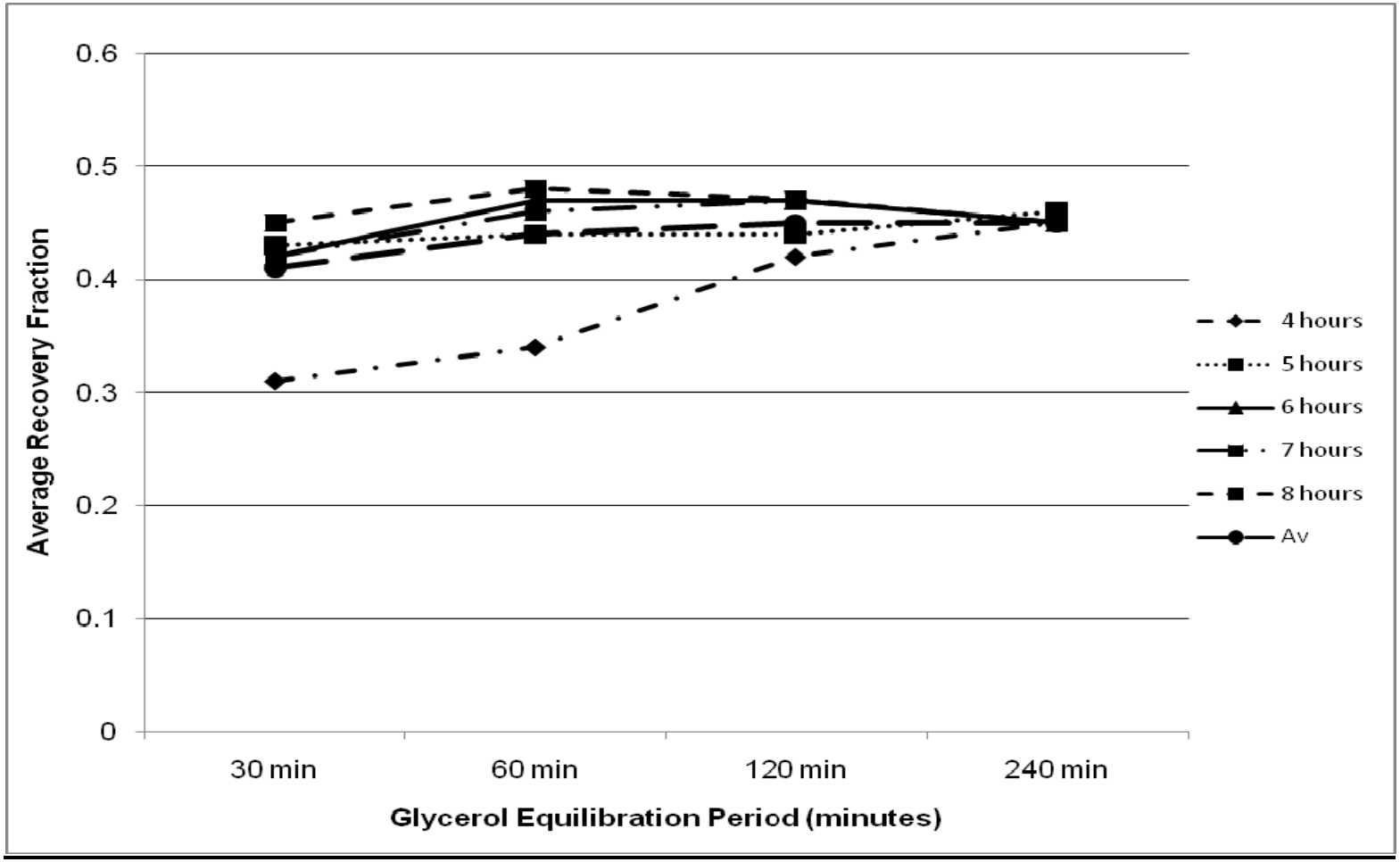
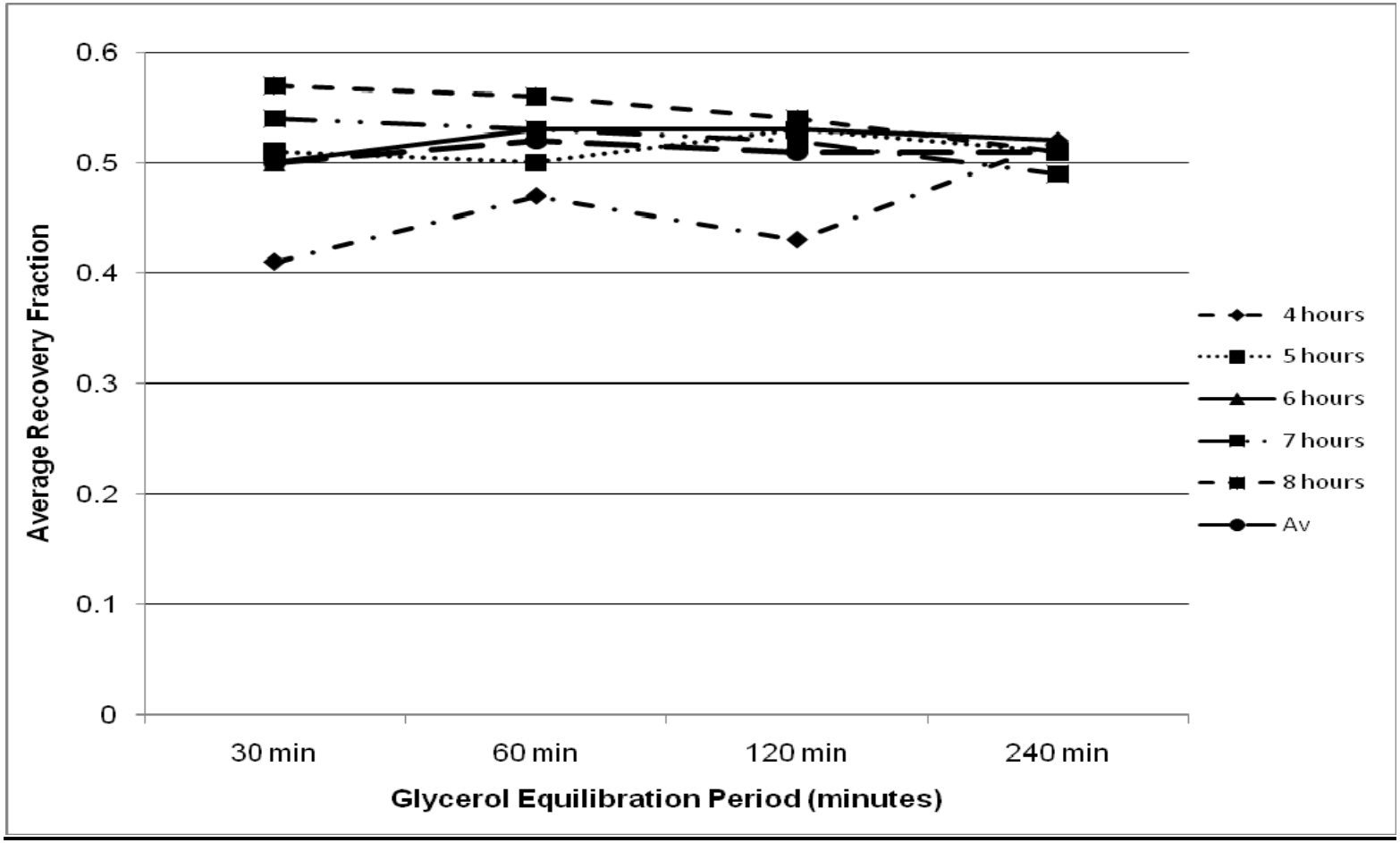


Figure 5.16: The relationship between glycerol equilibration period and mean recovery fraction of cryopreserved semen from Nguni bulls



From a practical perspective, a user-friendly matrix comparing the different combinations of cooling and glycerol equilibration periods studied in Experiment 2 would be of value to the semen cryopreservation industry. This cooling period and glycerol equilibration period matrix would then be a very helpful tool in managing the quality of cryopreserved semen for the African indigenous bulls.

5.11. Effect of different combinations of cooling and glycerol equilibration periods on the post-thaw sperm motility of bovine semen

Mean post-thaw sperm motility rates for cooling periods over all glycerol equilibration periods increased as the period of cooling increased. A 4 h cooling period resulted in a mean post-thaw motility rate of 35.7±10.4% while cooling periods of 5, 6, 7 and 8 h minutes recorded post-thaw motility rates of 40.7±9.7%, 41.2±8.5%, 41.5±10.5% and 42.8±9.8%, respectively. The mean post-thaw motility rate after a 4 h cooling period was significantly lower ($p<0.05$) than the mean post-thaw motility rates yielded after any of the longer cooling periods. The post-thaw motility rate after cooling periods of 5, 6, 7 or 8 h did not differ significantly ($p<0.05$ or $p<0.1$) from one another

Glycerol equilibration periods of 30, 60 and 120 minutes resulted in increasing (not significant) mean post-thaw motility rates of 38.9±11.6%, 40.6±10.3% and 41.1±9.2% while there was a slight decline (not significant) in the mean post-thaw motility (40.8±9.0) after a 240 minute glycerol equilibration period. Glycerol equilibration periods of 30, 60, 120 and 240 minutes yielded post-thaw motility rates that did however not differ significantly from one another.

The lowest mean rate of post-thaw motility (31.0±13.5%) was the result of combining a 4 h cooling period and a 30 minute glycerol equilibration period. This post-thaw motility rate did not differ significantly from the cooling period glycerol equilibration period combinations of 4 h 60 minutes or 4 h 120 minutes, which recorded the second and third lowest post-thaw sperm motility rates of 34.3±10.6% and 36.6±8.2%, respectively. The mean post-thaw motility resulting from a combination of 4 h cooling and 30 minutes

of glycerol equilibration however tended to differ from the post-thaw motility resulting after a 6 h 30 minutes cooling glycerol equilibration period combination. The mean post-thaw motility resulting from a combination of 4 h cooling and 30 minutes of glycerol equilibration also differed significantly from the post-thaw motility rates resulting from all other cooling glycerol equilibration period combinations.

The second lowest mean post-thaw motility rate (34.3±10.6%), yielded by the combination of 4 h cooling and 60 minutes glycerol equilibration, did not differ significantly from the mean post-thaw motility rates after cooling glycerol equilibration period combinations of 4h 30 minutes, 4 h 120 minutes, 6 h 30 minutes, 5 h 60 minutes, 7 h 240 minutes, 5 h 30 minutes, 8 h 240 minutes, 6 h 240 minutes, 4 h 240 minutes and 7 h 30 minutes. The mean post-thaw sperm motility yielded by the cooling glycerol equilibration period combination of 4 h 60 minutes tended to differ from mean post-thaw motility rates after cooling-glycerol equilibration period combinations of 5 h 120 minutes, 5 h 240 minutes, 7 h 60 minutes, 6 h 60 minutes, 7 h 120 minutes and 6 h 120 minutes. The mean post-thaw motility rate recorded by the cooling-glycerol equilibration period combination of 4 h-60 minutes differed from mean post-thaw motility rates after cooling-glycerol equilibration period combinations of 8 h 120 minutes, 8 h 30 minutes and 8 h 60 minutes.

The third lowest mean post-thaw motility rate (36.6±8.2%) was the result of combining 4 h of cooling with 120 minutes of glycerol equilibration. This rate tended to differ from the mean post-thaw sperm motility after the combination of 8 h cooling, followed by 60 minutes of glycerol equilibration. The third lowest mean post-thaw sperm motility rate was not significantly different from the mean post-thaw motility rates resulting from any of the remaining combinations of cooling glycerol equilibration period.

The combination of a 6 h cooling period, followed by a 30 minute glycerol equilibration period resulted in a mean post-thaw sperm of 39.1±8.8%. This rate tended to differ from the lowest mean post-thaw motility rate yielded by a 4 h cooling 30 minute glycerol equilibration period combination. There was no significant difference between the mean post-thaw motility from a 6 h 30 minute cooling glycerol equilibration period combination

and the mean post-thaw motility rates resulting from the remaining cooling glycerol equilibration period combinations.

Combinations of 5 h 60 minute, 7 h 240 minutes, 5 h 30 minutes, 8 h 240 minute, 6 h 240 minute, 4 h 240 minute, 7 h 30 minute, 5 h 120 minute, 5 h 240 minute, 7 h 60 minute, 6 h 60 minute, 7 h 120 minute, 6 h 120 minute, 8 h 120 minute, 8 h 30 minute and 8 h 60 minute cooling glycerol equilibration periods yielded mean post-thaw motility rates of 39.9±10.1%, 40.0±13.3%, 40.0±10.7%, 40.7±8.1%, 41.0±8.2%, 41.0±7.2%, 41.3±10.8%, 41.4±10.6%, 41.4±8.6%, 42.1±10.4%, 42.3±9.6%, 42.4±8.7%, 42.5±8.3, 42.8±10.1%, 43.3±12.0%, 44.4±9.8%, respectively. The above mentioned mean post-thaw motility rates did not significantly differ from one another.

TABLE 5.11: The overall effect of different combinations of cooling periods and glycerol equilibration periods on the mean (±SD) post-thaw sperm motility of bovine spermatozoa

		COOLING PERIOD (minutes)					
GLYCEROL EQUILIBRATION PERIOD (minutes)		**4**	**5**	**6**	**7**	**8**	**Overall mean (±SD)**
	30	31.0^{aA}±13.5	40.0^{bc}±10.7	39.1^{aBc}±8.8	41.3^{bc}±10.8	43.3^{c}±12.0	**38.9^{a}±11.6**
	60	34.3^{abA}±10.6	39.9^{bc}±10.1	42.3^{bBc}±9.6	42.1^{bBc}±10.4	44.4^{cB}±9.8	**40.6^{a}±10.3**
	120	36.6^{abcA}±8.2	41.4^{bBc}±10.6	42.5^{bBc}±8.3	42.4^{bBc}±8.7	42.8^{c}±10.1	**41.1^{a}±9.2**
	240	41.0^{bc}±7.2	41.4^{bBc}±8.6	41.0^{bc}±8.2	40.0^{bc}±13.3	40.7^{bc}±8.1	**40.8^{a}±9.0**
	Overall mean (±SD)	**35.7^{a}±10.4**	**40.7^{b}±9.7**	**41.2^{b}±8.5**	**41.5^{b}±10.5**	**42.8^{b}±9.8**	

^{a,b,c} Means with different superscript letters differ significantly ($p<0.05$)
$^{A.B}$ Means with different superscript letter tended to differ ($p<0.1$)

CHAPTER 6

General conclusion and recommendations

The results obtained in Experiment 1 on the effects of total equilibration period on post-thaw semen motility suggest that longer periods (10 to 12 h) yielded the highest post-thaw sperm motility rates and recovery fractions of semen. Overall, 11 h consistently yielded the highest post-thaw motility rates in both breeds, although the results did not differ significantly from the post-thaw motility or recovery fractions yielded after 10 or 12 h of total equilibration.

The general trend for the glycerol equilibration periods studied in Experiment 1 was that the resulting overall average post-thaw motility rates and recovery fraction increased with increasing periods. There was a breed difference when comparing the average post-thaw motility rates after 4, 5, 6 and 8 h ($p<0.05$), while the average post-thaw motility rates tended to differ after 7 h of equilibration.

Longer cooling periods also tended to increase the average post-thaw sperm motility rates and average recovery fractions in Experiment. Breed influenced the average post-thaw motility rates after 30 and 240 minutes ($p<0.05$), and at 120 minutes, the average post-thaw motility rates tended to differ between the Nguni and Boran breeds. After a cooling period of 240 minutes, the average recovery fractions of semen from the different breeds also differed significantly ($p<0.05$). A cooling period of 30 minutes resulted in average recovery fractions that tended to differ between the two breeds.

The above-mentioned trends demonstrate that if a short cooling period is applied, semen should be allowed longer glycerol equilibration periods – which in turn suggests a longer total equilibration time. This includes both the cooling and glycerol equilibration periods and should be applied to semen before freezing.

In Experiment 2, longer cooling periods were combined with shorter glycerol equilibration periods. When these periods were combined, the same set of total equilibration periods resulted. However, in Experiment 2 the highest average post-thaw motility rate and average recovery fraction resulted from 8 and 8.5 h of equilibration, respectively. In Boran bulls a 9 h total equilibration period recorded in numerically the highest (not significantly significant) average post-thaw sperm motility rate, as well as the average recovery fraction. A 7 h total equilibration period resulted in the highest (not significantly significant) average post-thaw motility rate and average recovery fraction for Nguni bulls.

The general trend observed for equilibration periods used in Experiment 2 was that the average post-thaw motility increased, as the glycerol equilibration period increased up to 120 minutes. However after 240 minutes of glycerol equilibration, there was a slight decline. The differences in average post-thaw motility after the respective glycerol equilibration periods were not significantly different. This trend mentioned above was also observed for both the Boran and Nguni bulls - although the differences in average post-thaw sperm motility were not significantly significant. There was however a breed difference ($p<0.05$) between the average post-thaw motility rates yielded by the two breeds, after each glycerol equilibration period. Overall, the average recovery fraction yielded by a 30 minute glycerol equilibration period was lower – although not significantly significant – than the average recovery fractions resulting from the other glycerol equilibration periods. For the Boran both the 120 and 240 minute glycerol equilibration periods resulted in the highest (significantly different from 30 minutes; $p<0.05$) average recovery fractions. A 60 minute glycerol equilibration period yielded the highest (not significantly different) average recovery fraction for the Nguni. There was again a breed difference ($p<0.05$) between the resulting average recovery fractions, after each glycerol equilibration period.

The average post-thaw sperm motility rates and average recovery fractions resulting from the different cooling periods studied in Experiment 2 showed a general trend to

increase with longer cooling periods. There were breed differences in the average post-thaw motility rates after 30, 60 and 120 ($p<0.05$) minutes, while a 240 minute cooling period resulted in average post-thaw motility rates that tended to differ between the breeds. Average recovery fractions of the different breeds also differed ($p<0.05$), after each of the cooling periods.

From the matrix to be used in practice, the best post-thaw sperm motility resulted from the combination of a 240 minute cooling period and a glycerol equilibration period of 6 to 8 h, if a shorter cooling period is desired (Experiment 1). If a longer cooling period is applied (Experiment 2), the highest post-thaw motility rate will most likely be achieved by a combination between an 8 h cooling period and 60 minute glycerol equilibration period.

For both Experiment 1 and Experiment 2, the null hypothesis – that there would be no significant differences between the post-thaw sperm motility or recovery fractions - is not accepted and the alternative hypothesis of significant differences between average post-thaw motility rates or average recovery fractions accepted.

CHAPTER 7

Critical evaluation

The results of the present study may have been more defined with a larger sample pool, but because of time and other practical restrictions, the amount of samples had to be limited.

The results of the present study confirm the results of some previous research and also explain some of the contradicting results of other research.

This study provides a great guideline for future studies to determine the optimum equilibration periods for African cattle breeds. It will be beneficial if other indigenous cattle breeds are included in future research on this topic.

CHAPTER 8

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APPENDIX A

DATA OBTAINED FROM EXPERIMENT 1

Bul no	Breed	Date	Cooling Time	Glycerol Equilibration Time	Initial Motility (%)	Post Thaw Motility (%)	Recovery Fraction	Environmental Temperature	Volume	Colour	Consistence	Mass Motility	Dilution	Room Temp	Total Time
3	N	19	4	0.5	90	30	0.33	25.6	7	4	4	3.5	12	24.8	4.5
6	N	19	4	0.5	85	40	0.47	25.1	11	3	4	3	9	23.3	4.5
7	N	13	4	0.5	80	20	0.25	22.8	7	3	3	2.5	7	22.1	4.5
10	N	19	4	0.5	90	30	0.33	25.9	4	3	3	3	10	26.1	4.5
8	N	19	4	0.5	90	60	0.67	25	4	3	3	3	9	25.4	4.5
3	N	19	4	1	90	45	0.50	25.6	7	4	4	3.5	12	24.8	5
6	N	19	4	1	85	38	0.45	25.1	11	3	4	3	9	23.3	5
7	N	13	4	1	80	35	0.44	22.8	7	3	3	2.5	7	22.1	5
10	N	19	4	1	90	35	0.39	25.9	4	3	3	3	10	26.1	5
8	N	19	4	1	90	50	0.56	25	4	3	3	3	9	25.4	5
3	N	19	4	2	90	45	0.50	25.6	7	4	4	3.5	12	24.8	6
6	N	19	4	2	85	40	0.47	25.1	11	3	4	3	9	23.3	6
7	N	13	4	2	80	20	0.25	22.8	7	3	3	2.5	7	22.1	6
10	N	19	4	2	90	35	0.39	25.9	4	3	3	3	10	26.1	6
8	N	19	4	2	90	50	0.56	25	4	3	3	3	9	25.4	6
3	N	19	4	4	90	45	0.50	25.6	7	4	4	3.5	12	24.8	8
6	N	19	4	4	85	50	0.59	25.1	11	3	4	3	9	23.3	8
7	N	13	4	4	80	40	0.50	22.8	7	3	3	2.5	7	22.1	8
10	N	19	4	4	90	35	0.39	25.9	4	3	3	3	10	26.1	8
8	N	19	4	4	90	55	0.61	25	4	3	3	3	9	25.4	8
3	N	19	5	0.5	90	40	0.44	25.6	7	4	4	3.5	12	24.8	5.5
6	N	19	5	0.5	85	46	0.54	25.1	11	3	4	3	9	23.3	5.5
7	N	13	5	0.5	80	32	0.40	22.8	7	3	3	2.5	7	22.1	5.5
10	N	19	5	0.5	90	35	0.39	25.9	4	3	3	3	10	26.1	5.5
8	N	19	5	0.5	90	68	0.76	25	4	3	3	3	9	25.4	5.5
3	N	19	5	1	90	50	0.56	25.6	7	4	4	3.5	12	24.8	6
6	N	19	5	1	85	38	0.45	25.1	11	3	4	4	9	23.3	6

7	N	13	5	1	80	32	0.40	22.8	7	3	3	2.5	7	22.1	6
10	N	19	5	1	90	33	0.37	25.9	4	3	3	3	10	26.1	6
8	N	19	5	1	90	65	0.72	25	4	3	3	3	9	25.4	6
3	N	19	5	2	90	55	0.61	25.6	7	4	4	3.5	12	24.8	7
6	N	19	5	2	85	45	0.53	25.1	11	3	4	3	9	23.3	7
7	N	13	5	2	80	35	0.44	22.8	7	3	3	2.5	7	22.1	7
10	N	19	5	2	90	33	0.37	25.9	4	3	3	3	10	26.1	7
8	N	19	5	2	90	65	0.72	25	4	3	3	3	9	25.4	7
3	N	19	5	4	90	50	0.56	25.6	7	4	4	3.5	12	24.8	9
6	N	19	5	4	85	45	0.53	25.1	11	3	4	3	9	23.3	9
7	N	13	5	4	80	32	0.40	22.8	7	3	3	2.5	7	22.1	9
10	N	19	5	4	90	35	0.39	25.9	4	3	3	3	10	26.1	9
8	N	19	5	4	90	60	0.67	25	4	3	3	3	9	25.4	9
3	N	19	6	0.5	90	45	0.50	25.6	7	4	4	3.5	12	24.8	6.5
6	N	19	6	0.5	85	46	0.54	25.1	11	3	4	3	9	23.3	6.5
7	N	13	6	0.5	80	35	0.44	22.8	7	3	3	2.5	7	22.1	6.5
10	N	19	6	0.5	90	30	0.33	25.9	4	3	3	3	10	26.1	6.5
8	N	19	6	0.5	90	60	0.67	25	4	3	3	3	9	25.4	6.5
3	N	19	6	1	90	55	0.61	25.6	7	4	4	3.5	12	24.8	7
6	N	19	6	1	85	50	0.59	25.1	11	3	4	3	9	23.3	7
7	N	13	6	1	80	35	0.44	22.8	7	3	3	2.5	7	22.1	7
10	N	19	6	1	90	30	0.33	25.9	4	3	3	3	10	26.1	7
8	N	19	6	1	90	60	0.67	25	4	3	3	3	9	25.4	7
3	N	19	6	2	90	55	0.61	25.6	7	4	4	3.5	12	24.8	7
6	N	19	6	2	85	42	0.49	25.1	11	3	4	3	9	23.3	8
7	N	13	6	2	80	40	0.50	22.8	7	3	3	2.5	7	22.1	8
10	N	19	6	2	90	35	0.39	25.9	4	3	3	3	10	26.1	8
8	N	19	6	2	90	60	0.67	25	4	3	3	3	9	25.4	8
3	N	19	6	4	90	50	0.56	25.6	7	4	4	3.5	12	24.8	10
6	N	19	6	4	85	43	0.51	25.1	11	3	4	3	9	23.3	10

7	N	13	6	2	80	38	0.47	22.8	7	3	3	2.5	7	22.1	8
10	N	19	6	4	90	33	0.37	25.9	4	3	3	3	10	26.1	10
8	N	19	6	4	90	60	0.67	25	4	3	3	3	9	25.4	10
3	N	19	7	0.5	90	55	0.61	25.6	7	4	4	3.5	12	24.8	7.5
6	N	19	7	0.5	85	44	0.52	25.1	11	3	4	3	9	23.3	7.5
7	N	13	7	0.5	80	35	0.44	22.8	4	3	3	2.5	10	22.1	7.5
10	N	19	7	0.5	90	38	0.42	25.9	4	3	3	3	10	26.1	7.5
8	N	19	7	0.5	90	65	0.72	25	4	3	3	3	9	25.4	7.5
3	N	19	7	1	90	50	0.56	25.6	7	4	4	3.5	12	24.8	8
6	N	19	7	1	85	48	0.56	25.1	11	3	4	3	9	23.3	8
7	N	13	7	1	80	35	0.44	22.8	4	3	3	2.5	10	22.1	8
10	N	19	7	1	90	33	0.37	25.9	4	3	3	3	10	26.1	8
8	N	19	7	1	90	65	0.72	25	4	3	3	3	9	25.4	8
3	N	19	7	2	90	55	0.61	25.6	7	4	4	3.5	12	24.8	7
6	N	19	7	2	85	40	0.47	25.1	11	3	4	3	9	23.3	9
7	N	13	7	2	80	35	0.44	22.8	4	3	3	2.5	10	22.1	9
10	N	19	7	2	90	38	0.42	25.9	4	3	3	3	10	26.1	9
8	N	19	7	2	90	60	0.67	25	4	3	3	3	9	25.4	9
3	N	19	7	4	90	50	0.56	25.6	7	4	4	3.5	12	24.8	11
6	N	19	7	4	85	45	0.53	25.1	11	3	4	3	9	23.3	11
7	N	13	7	4	80	35	0.44	22.8	4	3	3	2.5	10	22.1	11
10	N	19	7	4	90	20	0.22	25.9	4	3	3	3	10	26.1	11
8	N	19	7	4	90	65	0.72	25	4	3	3	3	9	25.4	11
3	N	19	8	0.5	90	55	0.61	25.6	7	4	4	3.5	12	24.8	8.5
6	N	19	8	0.5	85	50	0.59	25.1	11	3	4	3	9	23.3	8.5
7	N	13	8	0.5	80	35	0.44	22.8	4	3	3	2.5	10	22.1	8.5
10	N	19	8	0.5	90	38	0.42	25.9	4	3	3	3	10	26.1	8.5
8	N	19	8	0.5	90	70	0.78	25	4	3	3	3	9	25.4	8.5
3	N	19	8	1	90	55	0.61	25.6	7	4	4	3.5	12	24.8	9
6	N	19	8	1	85	52	0.61	25.1	11	3	4	3	9	23.3	9

7	N	13	8	1	80	38	0.48	22.8	4	3	3	2.5	10	22.1	9
10	N	19	8	1	90	35	0.39	25.9	4	3	3	3	10	26.1	9
8	N	19	8	1	90	65	0.72	25	4	3	3	3	9	25.4	9
3	N	19	8	2	90	55	0.61	25.6	7	4	4	3.5	12	24.8	10
6	N	19	8	2	85	40	0.47	25.1	11	3	4	3	9	23.3	10
7	N	13	8	2	80	40	0.50	22.8	4	3	3	2.5	10	22.1	10
10	N	19	8	2	90	35	0.39	25.9	4	3	3	3	10	26.1	10
8	N	19	8	2	90	65	0.72	25	4	3	3	3	9	25.4	10
3	N	19	8	4	90	45	0.50	25.6	7	4	4	2.5	12	24.8	12
6	N	19	8	4	85	41	0.48	25.1	11	3	4	3	9	23.3	12
7	N	13	8	4	80	40	0.50	22.8	4	3	3	2.5	10	22.1	12
10	N	19	8	4	90	35	0.39	25.9	4	3	3	3	10	26.1	12
8	N	19	8	4	90	60	0.67	25	4	3	3	3	9	25.4	12
A	B	19	4	0.5	85	10	0.12	25.1	7	3	4	2.5	10	23.9	4.5
B	B	19	4	0.5	80	35	0.44	26.3	7.5	2	3	2.5	7	26.6	4.5
C	B	34	4	0.5	85	30	0.35	25.8	6	3	5	3	12	24.5	4.5
D	B	34	4	0.5	80	20	0.25	25.6	3.5	2	3	2.5	6	25.1	4.5
E	B	30	4	0.5	85	35	0.41	31	8	3	5	3	11	22.4	4.5
A	B	19	4	1	85	15	0.18	25.1	7	3	4	2.5	10	23.9	5
B	B	19	4	1	80	35	0.44	26.3	7.5	2	3	2.5	7	26.6	5
C	B	34	4	1	85	30	0.35	25.8	6	3	5	3	12	24.5	5
D	B	34	4	1	80	20	0.25	25.6	3.5	2	3	2.5	6	25.1	5
E	B	30	4	1	85	40	0.47	31	8	3	5	3	11	22.4	5
A	B	19	4	2	85	35	0.41	25.1	7	3	4	2.5	10	23.9	6
B	B	19	4	2	80	38	0.48	26.3	7.5	2	3	2.5	7	26.6	6
C	B	34	4	2	85	30	0.35	25.8	6	3	5	3	12	24.5	6
D	B	34	4	2	80	33	0.41	25.6	3.5	2	3	2.5	6	25.1	6
E	B	30	4	2	85	40	0.47	31	8	3	5	3	11	22.4	6
A	B	19	4	4	85	35	0.41	25.1	7	3	4	2.5	10	23.9	8
B	B	19	4	4	80	35	0.44	26.3	7.5	2	3	2.5	7	26.6	8

C	B	34	4	4	85	33	0.39	25.8	6	3	5	3	12	24.5	8
D	B	34	4	4	80	42	0.53	25.6	3.5	2	3	2.5	6	25.1	8
E	B	30	4	4	85	40	0.47	31	8	3	5	3	11	22.4	8
A	B	19	5	0.5	85	33	0.39	25.1	7	3	4	2.5	10	23.9	5.5
B	B	19	5	0.5	80	40	0.50	26.3	7.5	2	3	2.5	7	26.6	5.5
C	B	34	5	0.5	85	33	0.39	25.8	6	3	5	3	12	24.5	5.5
D	B	34	5	0.5	80	35	0.44	25.6	3.5	2	3	2.5	6	25.1	5.5
E	B	30	5	0.5	85	38	0.45	31	8	3	5	3	11	22.4	5.5
A	B	19	5	1	85	35	0.41	25.1	7	3	4	2.5	10	23.9	6
B	B	19	5	1	80	38	0.48	26.3	7.5	2	3	2.5	7	26.6	6
C	B	34	5	1	85	35	0.41	25.8	6	3	5	3	12	24.5	6
D	B	34	5	1	80	35	0.44	25.6	3.5	2	3	2.5	6	25.1	6
E	B	30	5	1	85	38	0.45	31	8	3	5	3	11	22.4	6
A	B	19	5	2	85	35	0.41	25.1	7	3	4	2.5	10	23.9	7
B	B	19	5	2	80	38	0.48	26.3	7.5	2	3	2.5	7	26.6	7
C	B	34	5	2	85	35	0.41	25.8	6	3	5	3	12	24.5	7
D	B	34	5	2	80	38	0.48	25.6	3.5	2	3	2.5	6	25.1	7
E	B	30	5	2	85	35	0.41	31	8	3	5	3	11	22.4	7
A	B	19	5	4	85	35	0.41	25.1	7	3	4	2.5	10	23.9	9
B	B	19	5	4	80	35	0.44	26.3	7.5	2	3	2.5	7	26.6	9
C	B	34	5	4	85	38	0.45	25.8	6	3	5	3	12	24.5	9
D	B	34	5	4	80	44	0.55	25.6	3.5	2	3	2.5	6	25.1	9
E	B	30	5	4	85	40	0.47	31	8	3	5	3	11	22.4	9
A	B	19	6	0.5	85	35	0.41	25.1	7	3	4	2.5	10	23.9	6.5
B	B	19	6	0.5	80	35	0.44	26.3	7.5	2	3	2.5	7	26.6	6.5
C	B	34	6	0.5	85	35	0.41	25.8	6	3	5	3	12	24.5	6.5
D	B	34	6	0.5	80	35	0.44	25.6	3.5	2	3	2.5	6	25.1	6.5
E	B	30	6	0.5	85	35	0.41	31	8	3	5	3	11	22.4	6.5
A	B	19	6	1	85	40	0.47	25.1	7	3	4	2.5	10	23.9	7
B	B	19	6	1	80	40	0.50	26.3	7.5	2	3	2.5	7	26.6	7

C	B	34	6	1	85	35	0.41	25.8	6	3	5	3	12	24.5	7
D	B	34	6	1	80	38	0.48	25.6	3.5	2	3	2.5	6	25.1	7
E	B	30	6	1	85	40	0.47	31	8	3	5	3	11	22.4	7
A	B	19	6	2	85	40	0.47	25.1	7	3	4	2.5	10	23.9	8
B	B	19	6	2	80	40	0.50	26.3	7.5	2	3	2.5	7	26.6	8
C	B	34	6	2	85	35	0.41	25.8	6	3	5	3	12	24.5	8
D	B	34	6	2	80	38	0.48	25.6	3.5	2	3	2.5	6	25.1	8
E	B	30	6	2	85	40	0.47	31	8	3	5	3	11	22.4	8
A	B	19	6	4	85	35	0.41	25.1	7	3	4	2.5	10	23.9	10
B	B	19	6	4	80	38	0.48	26.3	7.5	2	3	2.5	7	26.6	10
C	B	34	6	4	85	35	0.41	25.8	6	3	5	3	12	24.5	10
D	B	34	6	4	80	40	0.50	25.6	3.5	2	3	2.5	6	25.1	10
E	B	30	6	4	85	38	0.45	31	8	3	5	3	11	22.4	10
A	B	19	7	0.5	85	30	0.35	25.1	7	3	4	2.5	10	23.9	7.5
B	B	19	7	0.5	80	38	0.48	26.3	7.5	2	3	3	7	26.6	7.5
C	B	34	7	0.5	85	35	0.41	25.8	6	3	5	2.5	12	24.5	7.5
D	B	34	7	0.5	80	33	0.41	25.6	3.5	2	3	3	6	25.1	7.5
E	B	30	7	0.5	85	40	0.47	31	8	3	5	2.5	11	22.4	7.5
A	B	19	7	1	85	40	0.47	25.1	7	3	4	2.5	10	23.9	8
B	B	19	7	1	80	35	0.44	26.3	7.5	2	3	3	7	26.6	8
C	B	34	7	1	85	30	0.35	25.8	6	3	5	2.5	12	24.5	8
D	B	34	7	1	80	45	0.56	25.6	3.5	2	3	3	6	25.1	8
E	B	30	7	1	85	40	0.47	31	8	3	5	2.5	11	22.4	8
A	B	19	7	2	85	40	0.47	25.1	7	3	4	2.5	10	23.9	9
B	B	19	7	2	80	33	0.41	26.3	7.5	2	3	3	7	26.6	9
C	B	34	7	2	85	38	0.45	25.8	6	3	5	2.5	12	24.5	9
D	B	34	7	2	80	45	0.56	25.6	3.5	2	3	3	6	25.1	9
E	B	30	7	2	85	40	0.47	31	8	3	5	2.5	11	22.4	9
A	B	19	7	4	85	40	0.47	25.1	7	3	4	2.5	10	23.9	11
B	B	19	7	4	80	40	0.50	26.3	7.5	2	3	3	7	26.6	11

C	B	34	7	4	85	20	0.24	25.8	6	3	5	2.5	12	24.5	11
D	B	34	7	4	80	40	0.50	25.6	3.5	2	3	3	6	25.1	11
E	B	30	7	4	85	45	0.53	31	8	3	5	3	11	22.4	11
A	B	19	8	0.5	85	35	0.41	25.1	7	3	4	2.5	10	23.9	8.5
B	B	19	8	0.5	80	44	0.55	26.3	7.5	2	3	2.5	7	26.6	8.5
C	B	34	8	0.5	85	38	0.45	25.8	6	3	5	3	12	24.5	8.5
D	B	34	8	0.5	80	30	0.38	25.6	3.5	2	3	2.5	6	25.1	8.5
E	B	30	8	0.5	85	38	0.45	31	8	3	5	3	11	22.4	8.5
A	B	19	8	1	85	40	0.47	25.1	7	3	4	2.5	10	23.9	9
B	B	19	8	1	80	38	0.48	26.3	7.5	2	3	2.5	7	26.6	9
C	B	34	8	1	85	38	0.45	25.8	6	3	5	3	12	24.5	9
D	B	34	8	1	80	45	0.56	25.6	3.5	2	3	2.5	6	25.1	9
E	B	30	8	1	85	38	0.45	31	8	3	5	3	11	22.4	9
A	B	19	8	2	85	40	0.47	25.1	7	3	4	2.5	10	23.9	10
B	B	19	8	2	80	38	0.48	26.3	7.5	2	3	2.5	7	26.6	10
C	B	34	8	2	85	30	0.35	25.8	6	3	5	3	12	24.5	10
D	B	34	8	2	80	45	0.56	25.6	3.5	2	3	2.5	6	25.1	10
E	B	30	8	2	85	40	0.47	31	8	3	5	3	11	22.4	10
A	B	19	8	4	85	33	0.39	25.1	7	3	4	2.5	10	23.9	12
B	B	19	8	4	80	35	0.44	26.3	7.5	2	3	2.5	7	26.6	12
C	B	34	8	4	85	33	0.39	25.8	6	3	5	3	12	24.5	12
D	B	34	8	4	80	45	0.56	25.6	3.5	2	3	2.5	6	25.1	12
E	B	30	8	4	85	40	0.47	31	8	3	5	3	11	22.4	12